

Growth Factors Involved In The Skeletal Metastases Of Prostate Cancer.

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ABSTRACT OF THESIS (Regulation 3.5.10)

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Prostate cancer selectively metastasises to the axial skeleton where it produces osteoblastic lesions.

Media conditioned by human osteoblast-like cells demonstrated growth stimulation of prostate cell lines in a dose dependent manner. PC-3 cells were stimulated to a maximum of 3.5 fold greater than control levels. DU145 cells and LNCaP were stimulated to a lesser extent, 2.3 ($p < 0.025$) and 1.5 fold ($p < 0.025$) respectively. This stimulatory activity was heat and acid sensitive and its production from the osteoblast-like cells was unaffected by 1nM dihydrotestosterone or 1nM $1,25(\text{OH})_2$ vitamin D.

Media conditioned by red bone marrow, derived from proximal femora, did not stimulate the growth of prostate cell lines. Prostate cell line growth was also unaffected by media conditioned by cell lines derived from bladder, lung and kidney (diluted to 50%), which represent other metastatic sites of prostate cancer.

To aid the characterisation of prostate mitogens in osteoblast-like cell conditioned medium, the effect of haematopoietic growth factors (produced predominantly in the bone environment) was tested. Results showed that PC-3 and DU145 could be significantly ($p < 0.0025$) stimulated by rEPO ($> 1\text{mIU/ml}$) and rGM-CSF ($> 0.1\text{IU/ml}$) in serum free medium. Stimulation was dose dependent. rEPO stimulated PC-3 cell growth 2.6 fold and DU145 2.2 fold. rGM-CSF stimulated growth of the two cell lines by a greater degree, PC-3 cells were stimulated 3 fold and DU145 2.3 fold. Growth of the same cell lines was unaffected by incubation with rIL-3 (0.1-1000mU/ml) or rG-CSF (0.1-1000IU/ml). The LNCaP cell line was stimulated only by rGM-CSF ($> 5\text{IU/ml}$).

To investigate further the involvement of GM-CSF in prostate cancer the presence of GM-CSF protein in the three prostate cell lines was determined by immunohistochemistry and examination of cell line conditioned media with ELISA and Western blotting. These techniques demonstrated that GM-CSF-like material was produced by DU145 and PC-3 cells but not by LNCaP. The results from ELISA found media conditioned by DU145 cells contained 1.7pg GM-CSF/ μg protein and PC-3 conditioned medium contained 2.5pg GM-CSF/ μg protein. The presence of GM-CSF gene transcripts in DU145 and PC-3 cells was established by reverse transcription and PCR of total RNA.

These findings suggest that osteoblast-like cell conditioned medium, GM-CSF and EPO may be responsible for the growth stimulation of prostate cancer in skeletal metastases. Production of GM-CSF by prostate carcinoma cells could play a role in the formation of osteoblastic lesions and may represent an autocrine mechanism of growth.



I dedicate my thesis to Harry Hoyle,
to his garden seat, Kitkats, smilers and chrysanthemums,
and also to my father, Bob Lang,
to his mountains, music, wine and Bob's Best.



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I Shona Lang, hereby declare that the work embodied within this thesis is the result of my own independent investigation. This is in accordance with rule 3.4.7 of the University of Edinburgh, Postgraduate Study Programme 1993/1994.

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Abbreviations:

aFGF	Acidic Fibroblast Growth Factor	³H-TdR	Methyl-tritiated Thymidine
ATP	Adenosine Triphosphate	HGF	Haematopoietic Growth Factor
bFGF	basic Fibroblast Growth Factor	HIFCS	Heat Inactivated Foetal Calf Serum
BM	Bone marrow	HPRT	Hypoxanthine Phosphoribosyl Transferase
bp	base pairs	IFN.γ	Interferon gamma
BPH	Benign Prostatic Hyperplasia	IGF-I, -II	Insulin-like Growth Factor type I, type II
BSA	Bovine Serum Albumin	IgG	Immunoglobulin type G
cAMP	cyclic Adenosine Monophosphate	IL-1: IL-7	Interleukin-1 : interleukin-7
CaP	Carcinoma of the Prostate	IU	International Units
cDNA	complementary DNA	Kd	Kilodaltons
Ci	Curies	M	Molar
CM	Conditioned Medium	(m)Ab	(monoclonal) Antibody
DAB	Diaminobenzidine	M-CSF	Macrophage-Colony Stimulating Factor
depc	diethyl pyrocarbonate	MHC	Major Histocompatibility
DHT	Dihydrotestosterone	ml	millilitre
DNA	Deoxyribonucleic Acid	mol	moles
dNTP	deoxynucleoside Triphosphates	mRNA	messenger Ribonucleic Acid
dpm	disintegrations per minute	MW	Molecular Weight
EGF	Epidermal Growth Factor	n	number of samples
ELISA	Enzyme Linked Immunosorbent Assay	OB	Osteoblast-like cell
EPO	Erythropoietin	OD	Optical Density
FCS	Foetal Calf Serum	PA(I)	Plasminogen Activator (Inhibitor)
g	gravity	PAGE	Polyacrylamide Gel Electrophoresis
G-CSF	Granulocyte-Colony Stimulating Factor	PCR	Polymerase Chain Reaction
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor		
GTC	Guanidine Isothiocyanate		

PDGF	Platelet Derived Growth Factor
POD	Peroxidase
pNP	para Nitrophenyl Phosphate
PTH	Parathyroid Hormone
r	recombinant
RNA	Ribose Nucleic Acid
RNAse	Ribonuclease
RT	Reverse Transcription
SA	Specific Activity
SCF	Stem Cell Factor
sd	standard deviation
SDS	Sodium Dodecyl Sulphate

SFM	Serum Free Medium
TBE	Tris Borate buffer
TBS	Tris Buffered Saline
TCA	Trichloroacetic Acid
TGFα	Transforming Growth Factor alpha
TGFβ	Transforming Growth Factor beta
TNF	Tumour Necrosis Factor
t-PA, u-PA	tissue type-, urokinase type- plasminogen activator.
U	Units
1,25 (OH)$_2$ Vitamin D	1 α , 25-dihydroxycholecalciferol

CHAPTER 1

INTRODUCTION

Prostate cancer is a slow growing tumour, the incidence of which is closely correlated to age (Silverberg 1987). A small percentage of these tumours become metastatic (Berretoni & Carter 1986), and this aggressive form of the disease has a high level of mortality since the metastases are usually multiple and destructive. Paget (1889) was the first to observe that cancers metastasise preferentially to selective secondary organs. He hypothesised that the environment of that secondary organ provided a 'nutritious soil' for the cancer to grow in. This could be explained by the presence of specific growth factors secreted by the secondary organ. The aim of this thesis was to identify growth factors secreted by the bone environment that can stimulate prostate cancer growth.

1.1 Prostate Cancer

Prostate cancer is now the second most common cause of cancer for males in the European Community (Jensen *et al* 1990), and a major cause of death for men over the age of 55 years. The cost of treatment is an ever growing burden on the State and with life expectancy increasing the problem will get worse. At the moment a successful treatment for advanced prostatic carcinoma has not been established. An understanding of the normal physiology and function of the prostate is essential to investigate how malignancy may arise.

a) Normal Physiology and Function Of The Prostate

The prostate is a musculoglandular organ, surrounding the prostatic urethra. Morphological and embryological studies by McNeal (1972, 1988a) have led to the identification of three main zones in the prostate gland (Figure 1):

- a) the peripheral zone
- b) the central zone
- c) the transitional zone

The peripheral zone is responsible for 70% of the glandular mass of the gland and the central zone a further 25%. Each zone has a separate composition of stroma, muscle fibre, accessory cells and a population of endocrine-paracrine cells. All possess ductal-acinar systems, lined with columnar, secretory epithelia (McNeal 1988a). The epithelia produce prostatic fluid which slowly collects in the gland. During emission this fluid injects into the urethra by muscular contraction, where it combines with spermatozoa and other seminal fluids. Prostatic fluid represents roughly 30% of the average ejaculate volume (Aumüller 1992). The main proteins found in the fluid are prostatic acid phosphatase (Lin *et al* 1986), prostatic specific antigen (Wang *et al* 1979) and β -microseminoprotein (Akiyama *et al* 1985). Citrate is also present, creating a pH of 6.5, which is thought to neutralise the alkalinity of cervical mucus. Other possible functions of the prostatic fluid include liquefaction of seminal fluid (Amelar 1962), sperm motility (by an ion-exchange reaction) and an immunosuppressive effect on the urethra or cervix (Aumüller 1992).

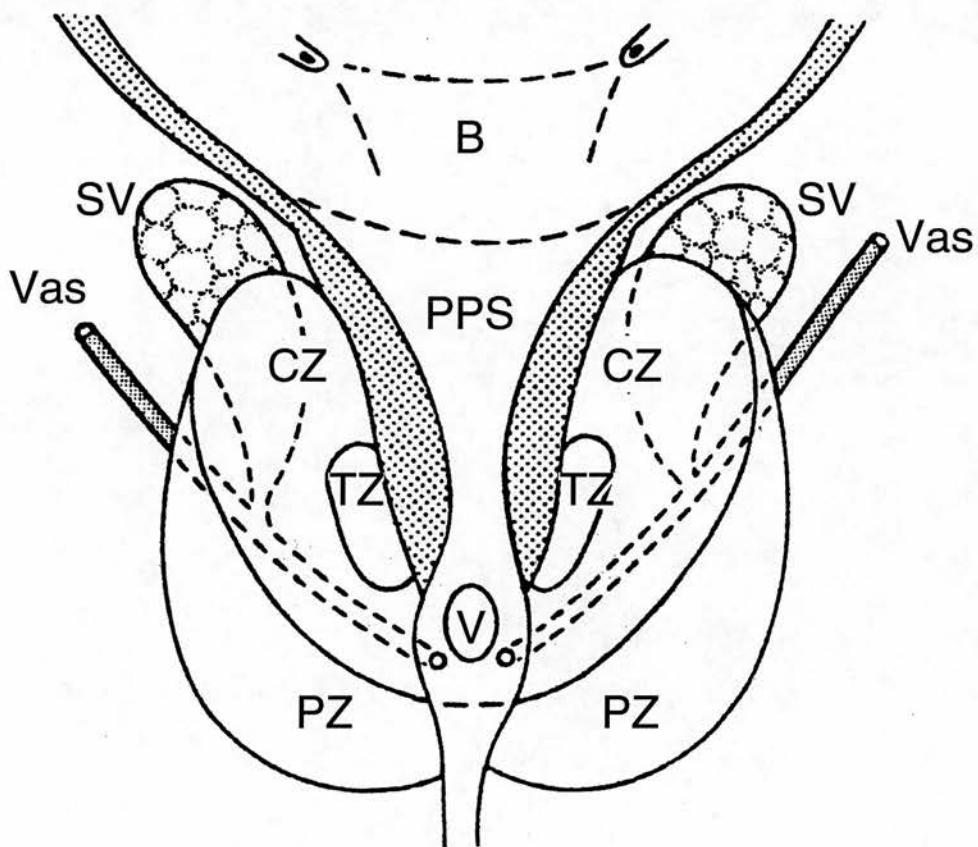
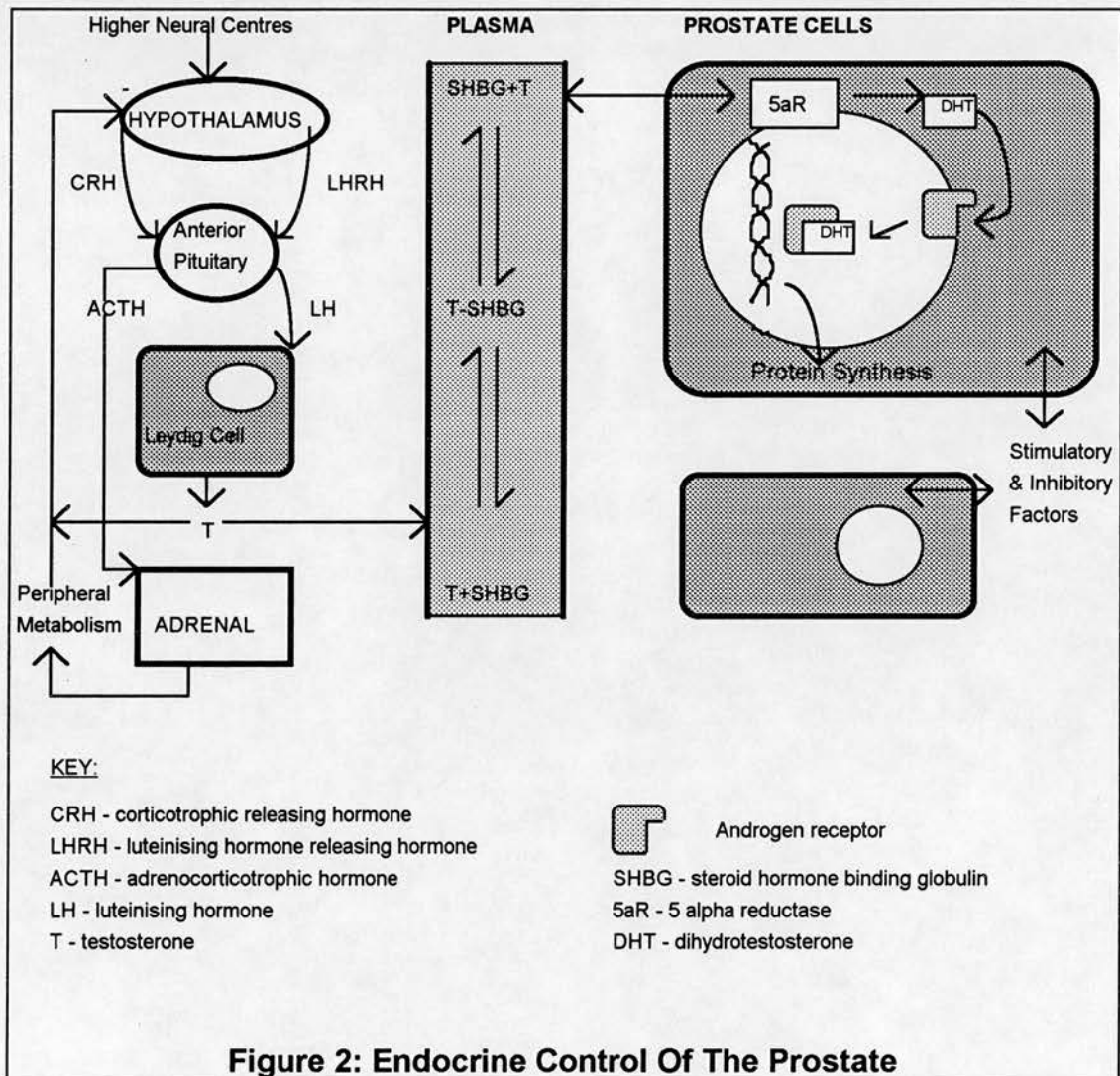


Figure 1: Diagram of adult prostate. Illustrating position of peripheral zone (PZ), central zone (CZ), transitional zone (TZ), pre-prostatic sphincter (PPS), verumontanum (V), seminal vesicles (SV), vas deferens (Vas) and bladder (B).

b) Regulation of Prostate Growth

Testicular androgens are essential for the normal functioning of the prostate. 95% of circulating testosterone is produced in the testes, the remaining 5% derives from adrenal androgens (Baird *et al* 1969). Testosterone production from both sources depends on the hypothalamic-pituitary-gonadal axis, illustrated in figure 2.



Testosterone is synthesised by the Leydig cells of the testes in response to the secretion of luteinising hormone from the anterior pituitary gland. The production of luteinising hormone is controlled by the pulsatile secretions of luteinising hormone releasing hormone (LHRH) from

hypothalamic neurones. The pulsatile nature of LHRH conveys a similar rhythmic, secretory nature to luteinising hormone (Clarke & Cummins 1987). Testosterone acts in a negative feedback loop on both the pituitary and the hypothalamus. This is achieved by decreasing the pulse frequency and amplitude of luteinising hormone secretion (Johnson and Everitt 1988). Integration of neural and endocrine systems occurs in the higher centres of the brain, where the secretion of LHRH can be regulated by stimulatory and inhibitory neurotransmitters e.g. catecholamines, serotonin and opioid systems (Clarke & Cummins 1987).

The production of adrenal androgens is dependent on the secretion of adrenocorticotrophic hormone from the pituitary, again, under the influence of the hypothalamus. These adrenal androgens can be metabolised by the prostate (Harper *et al* 1974).

90% of the total circulating testosterone is bound to serum proteins (albumin and steroid hormone binding globulin), the remaining 10% is unbound and therefore available to the prostate cell (Peeling & Griffiths 1987). This available testosterone is taken into the prostate cell and reduced by the enzyme 5 α -reductase to form dihydrotestosterone (DHT). 5 α -reductase is found on the nuclear membrane, endoplasmic reticulum and in smaller quantities in the cytoplasm (Moore *et al* 1974), though it may be confined to the nuclear membrane (Houston *et al* 1985). DHT binds to the hormone binding region of the androgen receptor, a protein with both hormone and DNA binding sites. This leads to conformational changes within the receptor which expose the DNA binding sites. The hormone-receptor complex can then attach to specific DNA sequences (androgen response elements) in the genome (Claessens *et al* 1989). Transcriptional

alterations are produced, which control further gene activity and protein production, essential for prostate growth (Katz *et al* 1989).

In peripheral tissues such as adipose, testosterone can be converted to oestradiol by aromatization (Folkerd & James 1983). Oestrogens have been found in the prostate (Ghanadian & Puah 1981) though whether or not oestrogen receptors are present in the prostate remains undetermined (F.K. Habib 1990). Prostate growth is also stimulated by prolactin, apparently due to a synergistic action with luteinizing hormone, which results in enhanced testosterone production (Hafiez *et al* 1972). Its stimulatory effect may also occur by potentiating 5 α -reductase activity (Yamanka *et al* 1975).

The normal growth of prostate is also dependent on locally produced growth factors. Stroma and epithelium are found in close proximity to each other within the prostate gland (Rohr & Bartsch 1980, McNeal 1988a). These cell types are thought to closely interact by the release of paracrine growth factors. This is illustrated by the research of Kabalin *et al* (1989) who showed that fibroblasts can stimulate epithelial cell colony growth. The paracrine interaction is further illustrated by the findings of Sugimura *et al* (1986) who showed that the growth of foetal epithelium is androgen-dependent only when androgen receptors are present in the mesenchyme, and not in the epithelium. Androgen receptors and 5 α -reductase are found in both epithelial and stromal compartments (Schweikert *et al* 1985, Kyprianou & Davies 1986), indicating both cell types could be affected by androgens. Table 1 indicates the increasing list of growth factors synthesised by the prostate.

Table 1: Prostate Derived Growth Factors

EGF	6Kd	(h)	(Maddy et al 1987, Jacobs et al 1988, Fowler et al 1988)
bFGF	16Kd	(h)	(Mydlo et al 1988, Story et al 1989)
IGF-II	7.5Kd	(r)	(Matuo et al 1988)
PDGF	30Kd	(h)	(Sitaras et al 1988)
TGF α	6Kd	(h)	(Wilding et al 1989a)
TGF β 1	25Kd	(r)	(Steiner et al 1991, Matuo et al 1990)
TGF β 2	25Kd	(h)	(Mori et al 1990)
Growth Hormone	20Kd	(h)	(Sibley et al 1984)
Prostate derived growth factor	25Kd	(r)	(Maehma et al 1986)
Nerve Growth Factor	26Kd	(h)	(Djakiew et al 1991)

(h-human, r-rat model)

Some of these factors are potentially responsible for the paracrine growth control discussed. Table 2 lists the variety of growth factors (stimulatory and inhibitory) known to affect the prostate.

Table 2: Prostate Growth Factors

STIMULATORY	INHIBITORY
Androgens (h) (Schuurmans et al 1988)	TGF β 25Kd (m) (Fernandez et al 1986)
Insulin 6Kd (h) (McKeehan et al 1987)	Prostate epithelium inhibitory factor 10-20Kd (Konig 1987)
Prolactin 23/48Kd (h) (Hafiez et al 1972)	
EGF 6Kd (h) (Schuurmans et al 1988, MacDonald et al 1990)	
bFGF 16Kd (h) (Story et al 1989)	
IGF-I 7Kd (h) (Fiorelli et al 1991)	
TGF α 6Kd (h) (Wilding et al 1989a)	
Nerve growth factor 26Kd (h) (Djakiew et al 1991)	

(h-human, m-mouse model)

Generally the mitogenic influences of EGF and FGF are balanced by the inhibitory activity of TGF β . EGF is both produced by and stimulates the prostate. The presence of its receptors have been demonstrated in normal prostate tissue (Fekete *et al* 1989), benign prostate (Maddy *et al* 1987), cancer tissue (Fekete *et al* 1989) and cell lines (MacDonald *et al* 1990). Studies on a prostate tumour cell line have demonstrated how EGF receptor expression can be increased in response to androgen stimulation (Schuurmans *et al* 1991), thus illustrating how growth is controlled by steroid and growth factor interactions.

The interactions of prostate cells, growth factors and hormones are clearly important for regulating growth of the normal gland. Progression of malignant growth is likely to be accompanied by a breakdown in these regulatory processes and an understanding of this may help find therapies.

c) Prostate cancer

Carcinomas of the prostate arise mainly in the peripheral zone of the gland (McNeal *et al* 1988b). The tumour grows towards and outside the prostatic capsule. This initial growth is slow and clinically unnoticed. In many cases the disease remains as a small focus and causes no medical problems (Carter & Coffey 1990). McNeal *et al* (1986) suggested 1ml is a critical size, with subsequent growth leading to biological aggressiveness and metastasis. The metastatic spread occurs through the lymphatics and the venous systems, with secondaries occurring in lymph nodes, bone and lungs (Busuttil 1990). Not until the cancer enlarges or metastasises does it become necessary to treat. Early forms of the cancer are hormone sensitive and if confined to the prostate can be treated with endocrine therapy, surgery, or irradiation. However 75% of patients diagnosed already have

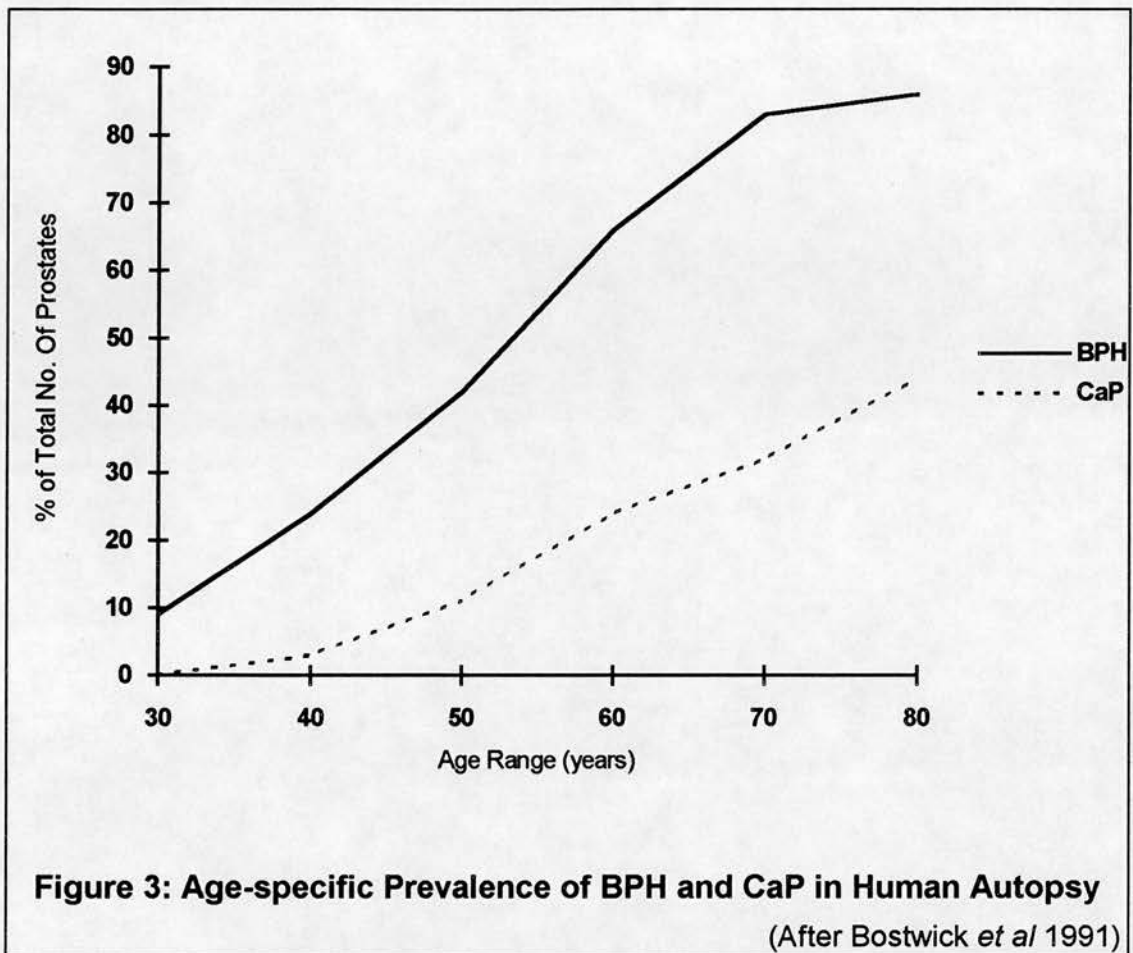
metastases (Ritchie 1989) which places them beyond the effectiveness of such treatments to fully remove the cancer (Ritchie 1989). Once the cancer progresses to a hormone insensitive state endocrine treatments cannot slow further progression and radiotherapy or chemotherapy are used to try and inhibit cancer growth (Nagel & Kölln 1977, Laing *et al* 1991). Skeletal metastases are usually very painful and interfere with surrounding musculature and neural structures. They also cause weakening of the bone leading to fractures and disrupt the bone marrow (Berrettoni and Carter 1986). Bisphosphonate compounds are used to alleviate the pain of skeletal metastases (Adami *et al* 1985, Yu-Cheng *et al* 1992) but they also do not present a cure, only slow progression.

Clearly preventative treatment is important for prostate cancer. Screening men would help find the curable, early primary tumours. Since men presented at diagnosis already have metastases such a move would be greatly beneficial.

i) Factors Involved In Prostate Cancer Formation

Various factors are involved in the formation of prostate carcinoma. The most striking of these is age. Figure 3 illustrates how the incidence of both benign prostatic hyperplasia (BPH) and prostate carcinoma (CaP) increases with age.

The incidence of benign prostatic hyperplasia increases from 20% to 80% between the ages of 40 and 80 years. Carcinoma is rarely seen before the age of 40 and its incidence lags behind benign prostatic hyperplasia in years (Bostwick *et al* 1991). The second most striking factor is the involvement of androgens. Anti-androgens and castration can inhibit hormone sensitive cancers (Tetu *et al* 1991, Huggins *et al* 1941), indicating that androgens are required for the growth of early tumours.



Epidemiological studies have indicated an environmental factor. The incidence of prostate cancer is much lower in Japan and China (0.1-0.3%) compared to the USA (12%). However Chinese and Japanese emigrants to the USA show an increase in incidence (Carter & Coffey 1990). This may be due to changing diet and certain studies have demonstrated a strong association between cancer and dairy products (Armstrong & Doll 1975).

The cancer can also be an inherited disease (Steinberg *et al* 1990). Evidence shows the risk for a man increases with number of affected relatives and how genetically close he is to an affected relative.

To investigate the mechanisms of prostate cancer formation and progression experimental models are required.

ii) Experimental Models

Various human and animal models exist for studying prostate carcinoma. An ideal model system should represent the human disease exactly. Therefore these models must be capable of eliciting responses to hormonal and chemotherapeutic manipulations, analogous to the natural disease. They should be capable of forming metastases and also create reproducible results. A major problem of producing such a system is that the fundamental prostate biology is not completely understood.

Animal Models

The most well known animal model is the Dunning rat tumour, a spontaneous tumour of the rat prostate (Lubaroff *et al* 1980). It is a useful model but limited because it isn't human nor does it form skeletal metastases (without artificial assistance). Rats, mice and hamsters are often used as hosts for the injection of human tumour tissue. This allows tumour progression and metastasis to be observed and manipulated. Sufficient numbers of animals can be used, allowing better statistical analysis of the results. The injection of human tissue can, however, lead to immunological reactions within the animal, which can complicate results. Hormone insensitive cells have been successfully injected into immune suppressed mice (Mickey *et al* 1980) but hormone sensitive cells have had low acceptance rates (Otto *et al* 1988).

Organ Culture

This technique employs whole organs cultured in tanks of culture medium or organ fragments cultured on plasma clots in petri dishes (Lasnitzki *et al* 1982). It allows functional and structural tissue preservation, and lasts for a few hours or days. Often the organ retains its hormonal responsiveness and secretory activities. Therefore this model allows a more

physiological investigation, though the lack of systemic influences means the model is still not true. McMahon *et al* (1972) successfully produced prostate organ cultures. This allowed drug response experiments on an organ from a patient with a known and useful medical history. However obtaining suitable positive and negative controls can be difficult. This problem is compounded by inconsistent availability of organs. Surgery may also cause damage or infection of the organ and once in culture deterioration occurs within days.

Primary Culture

The problems of organ culture can be overcome with primary cultures - cells cultured from fresh tissue. An isolated cell system allows the investigation of specific changes in carcinogenesis (a difficult task *in vivo*). Primary cultures provide a means of isolating fibroblasts and epithelial cell cultures, usually employing collagenase digestion (Chaproniere *et al* 1986, Peehl 1985). These cells are representative of the original organ because they are not altered by prolonged *in vitro* serial culture. The technique allows improved reproducibility when compared to organ culture, though the cultured cells have a finite life span and serial dilution is not always possible. They are still prone to bacterial and fungal contamination. It was discussed earlier that the normal growth of the prostate is, in part, regulated by paracrine interactions between the fibroblast and epithelial cells. Loss of this interaction may result in unrepresentative findings. Epithelial cells will generally be contaminated with fibroblasts, and malignant cultures contaminated with sub-populations of benign cells. Therefore, exact characterisation of the culture is difficult to determine. These latter problems are overcome using cell lines.

Cell Lines

Established cell lines have the ability to sub-culture indefinitely *in vitro*. The 'infinite' life of these cells is their major advantage, allowing

greater accuracy for experimental repetition than any other model. It is unknown if these cells are immortal due to a series of mutations in culture (Periera-Smith & Smith 1988) or the clonal expansion of an immortal cell from the original neoplastic tissue. Diverging characteristics of the same cell line can result from long-term culture of cloned cells, especially if culture conditions vary between different laboratories (Labrie & Veilleux 1986). One cell line can be easily contaminated with another cell line if tissue culture technique is poor. Results therefore, may not be reproducible between different laboratories. Bacterial and fungal contaminations present fewer problems than other models, however the imperceptible contamination of mycoplasma is a greater concern. Mycoplasma is difficult to detect and can affect cell growth, thereby negating experimental results (McGarrity *et al* 1985).

Prostate cell lines are available which represent pure cultures of malignant, epithelial prostate cells, in both hormone sensitive and insensitive stages of malignancy. The cell line LNCaP is hormone sensitive, showing increased growth when stimulated with DHT (Horoszewicz *et al* 1983, Hasenson *et al* 1985). It is derived from a supraclavicular lymph node metastatic lesion. Many aspects of functional differentiation are preserved such as prostatic acid phosphatase, prostate specific antigen and androgen receptors in the cytosol and nuclear fractions. The cell lines DU145 and PC-3 are both hormone insensitive. DU145 was established from a brain metastasis (Stone *et al* 1978, Mickey *et al* 1980) and PC-3 from a bone metastasis (Kaighn *et al* 1979). Both exhibit fewer prostatic markers than LNCaP with prostatic acid phosphatase present at low levels and androgen receptors expressed at very low levels or not at all (Tilley *et al* 1990). PC-3

and DU145 grow in very low serum and with a faster growth rate than LNCaP (for a summary of properties see table 3).

Table 3: Characteristics of prostate carcinoma cell lines

CELL LINE	DU145	PC-3	LNCaP
ORIGIN	Brain metastasis	Bone metastasis	Lymph node metastasis
CELL TYPE	epithelia	epithelia	epithelia
HORMONE SENSITIVE	no	no	yes
FORM TUMOURS IN NUDE MICE	yes	yes	yes
ACID PHOSPHATASE	low	low	high
REDUCED SERUM DEPENDENCE	yes	yes	no
DOUBLING TIME (hrs)	34	33	60
ANDROGEN RECEPTOR	no	no	yes

The three cell lines represent useful models for prostate cancer, a disease that is originally hormone responsive but eventually becomes unresponsive. LNCaP cells represent the early, hormone responsive stages of prostate cancer and DU145 and PC-3 cell lines represent later poorly differentiated stages of the cancer that are hormone insensitive and more malignant.

These models have allowed a variety of investigations into the study of prostate cancer tumorigenesis, allowing basic cellular and molecular research.

1.2 Malignancy and Growth Factors

Tumour cells have abnormal growth patterns and are unresponsive to the normal mechanisms of growth control. The aberrant presentation, production or mutation of a growth factor can lead to this escape from orderly growth.

a) Normal Cell Growth

Control of growth is one of the most important and least understood areas of biology. It is a precise and carefully regulated process allowing an organ to grow to a specific size and combination of cell types. A balance of inhibitory and stimulatory factors are required, together with various differentiation factors.

A cell is either quiescent or growing. If the cell is growing it undergoes a series of phases known as the cell cycle, illustrated in figure 4:

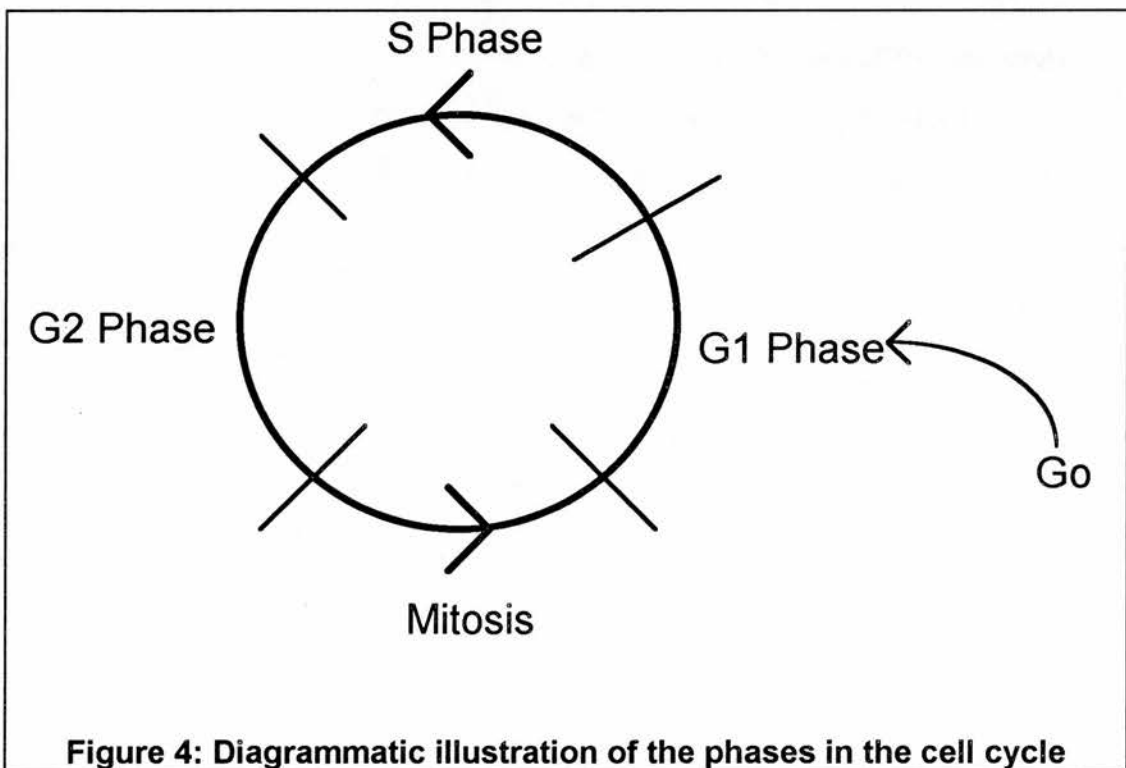


Figure 4: Diagrammatic illustration of the phases in the cell cycle

During the cell cycle, there is an increase of mass, DNA division and finally cell division. G_1 , G_2 and S phase comprise interphase during which there is cellular growth and synthesis of cellular proteins and other macromolecules. In G_1 , cells commit themselves to initiate DNA synthesis and the DNA content is then doubled in S phase. In G_2 , chromatin condenses in preparation for mitosis (M phase) which results in the formation of two daughter cells. A quiescent cell is classified as G_0 . A cell in G_0 may be resting or terminally differentiated (e.g. nerve cells) (Rees & Steinberg 1984).

The complicated regulation of cell division depends on correctly co-ordinating gene transcription, protein translation and protein function. Growth factors and hormones play an important role in this co-ordination.

b) Growth Factors

Growth factors control the growth and differentiation of cells, by providing a basic level of inter- and intracellular communication, predominantly within local cellular environments. They are polypeptide molecules with varying degrees of glycosylation and can be synthesised by a variety of cells. In contrast, hormones are produced by a specialised gland and have endocrine actions. Growth factor action is either autocrine or paracrine (see figure 5).

Hormones are described as endocrine since they are synthesised in organs at distant sites to their target cell and then transported from one to the other in the circulation (e.g. testosterone). Growth factor action is usually local and communicates between two cells (paracrine) or exerts its action on the cell which synthesised it (autocrine).

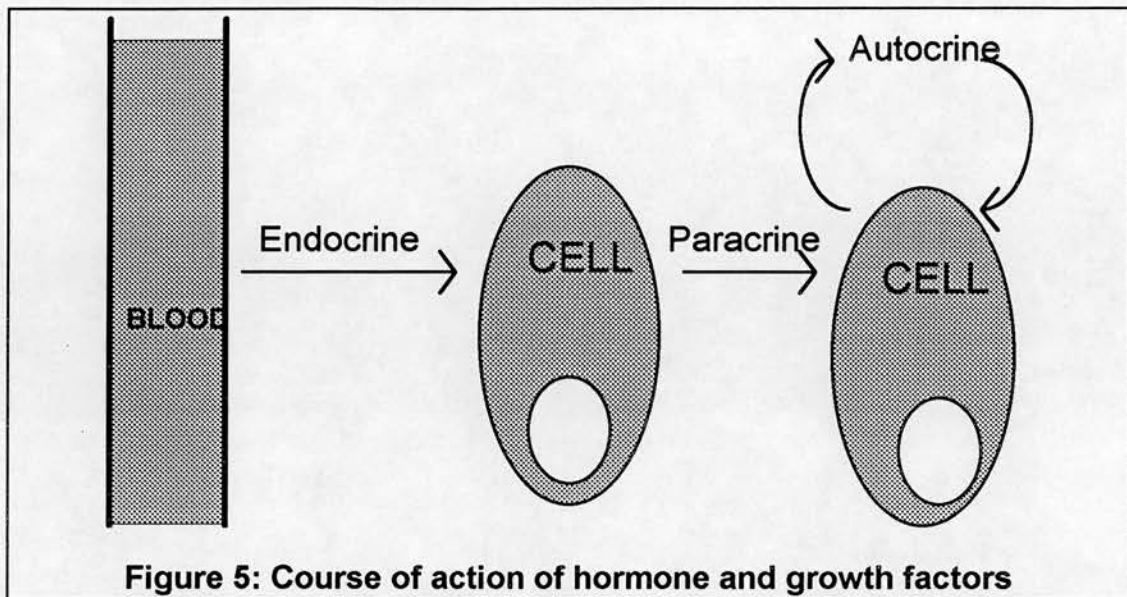


Figure 5: Course of action of hormone and growth factors

i) Common Growth Factors

The most commonly known growth factor is epidermal growth factor (EGF). It is a single polypeptide chain of 53 amino acids and molecular weight, 6Kd. EGF was named due to its ability to cause proliferation of epidermal and epithelial tissues, however it can stimulate and differentiate a wide variety of cells (Carpenter & Cohen 1979). Similar in structure to EGF is transforming growth factor alpha ($TGF\alpha$). It too is a small polypeptide of 50 amino acids, synthesised from a larger precursor. $TGF\alpha$ can compete for the EGF receptor and produce all the effects of EGF (Winkler *et al* 1989), and in some circumstances can be more potent.

Transforming growth factor beta ($TGF\beta$) belongs to a large family of molecules that share gene homology. The family includes inhibins and bone morphogenetic proteins (Lyons & Moses 1990). Despite its name, $TGF\beta$ has no similarity to $TGF\alpha$. $TGF\beta$ is a 25Kd dimeric protein, synthesised from a larger precursor. There are four types of $TGF\beta$ (three in humans), which share similar biological activities and gene homology. As a whole they have

diverse biological activities over a wide range of tissues, these include: chemotaxis, growth inhibition and extracellular matrix production (Lyons & Moses 1990).

The fibroblast growth factors (FGF) exist in acidic and basic forms. Although produced from different genes they are closely related and have similar biological activities, both with a molecular weight of 16Kd (Gospodarowicz *et al* 1987). They are multifunctional with the widest range of stimulatory and differentiating activities of all the growth factors, and are well known for their potent angiogenic functions. Platelet derived growth factor (PDGF) is the major mitogen of connective tissues and is important for wound healing. It is a 30Kd dimer and the products of two genes. The two peptide chains have 60% amino acid homology (Heldin *et al* 1985).

The last of the common growth factor families are the insulin-like growth factors (IGF). There are two members of this family; IGF-I (somatomedin C) and IGF-II (skeletal growth factor), which have a molecular weights of 7.5Kd. They share structural (the insulin fold) and functional similarities to insulin (Dafgard *et al* 1985).

The above gives a general indication of the effects of the common growth factors. Although growth factors are often named after the cell type they were first identified to affect, their actions are often more widespread and complex than those listed. The effect of one growth factor will depend on the other growth factors present and their interactions together (Sporn & Roberts 1988). For example the stimulatory effects of EGF on prostate cell lines can be increased by androgens and decreased by TGF β (Schuurmans *et al* 1988). The biological effects of TGF β vary (i.e., it can be stimulatory or inhibitory) depending on which other growth factors are present (Lyons & Moses 1990).

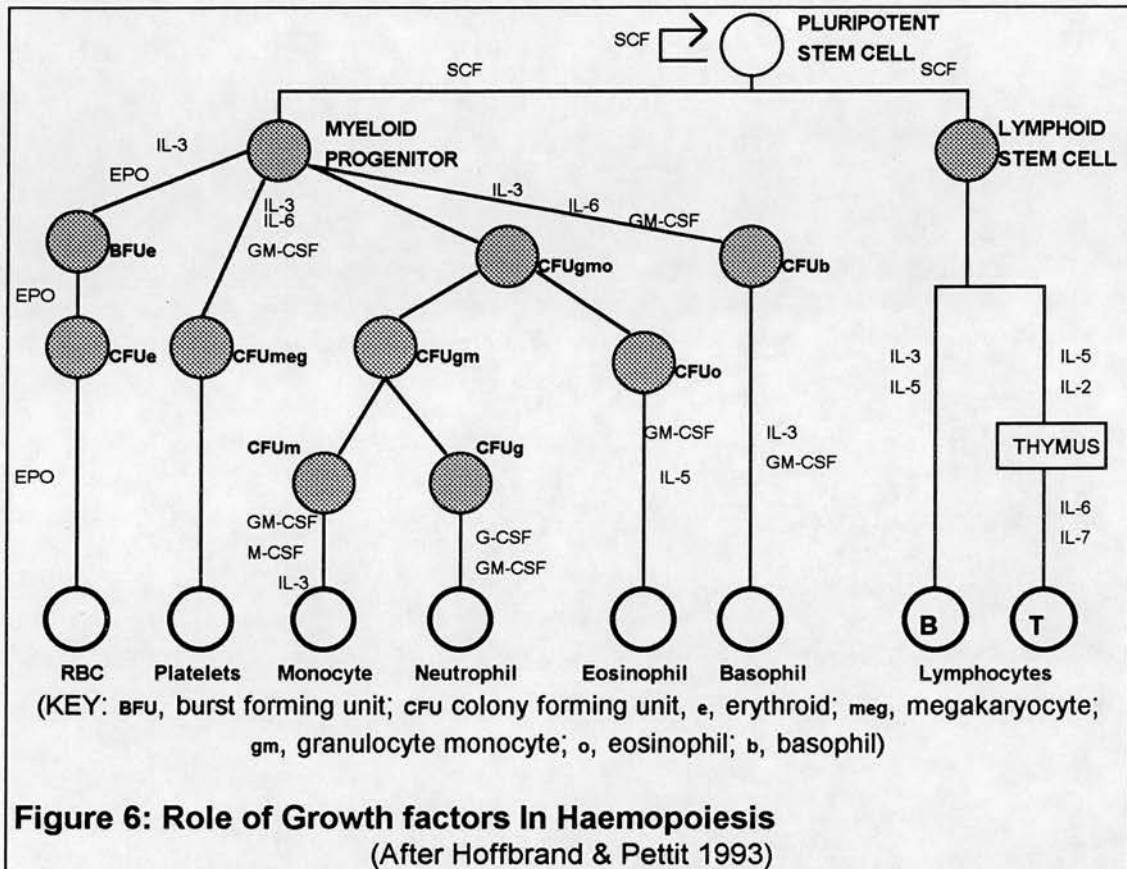
Recent studies have led to the discovery of cytokines, a group of growth factors involved in inflammatory and immune processes. It includes common factors such as TGF β and the more recently identified factors; interleukins and colony stimulating factors. Within the group is another set of growth factors, the haematopoietic growth factors, which control the growth and differentiation of haematopoietic cells. Much of this thesis will look at haematopoietic growth factors in more detail since they are likely candidates for stimulating prostate cancer in skeletal sites.

ii) Haematopoietic Growth Factors

Haematopoietic growth factors are glycoproteins specifically involved in the survival, differentiation and function of cells from the haematopoietic system. Most of these factors are interleukins or colony stimulating factors. They can be produced by T-lymphocytes, monocytes, endothelial cells and fibroblasts. The exception to this is erythropoietin which is a hormone produced by the kidney in response to hypoxia (Hoffbrand & Pettit 1993). Erythropoietin stimulates the maturation of red blood cells allowing increased oxygen delivery. Levels of haematopoietic growth factors are low or undetectable except during inflammation.

Haematopoietic cells (platelets, lymphocytes, erythrocytes, monocytes, macrophages and granulocytes) may have very different morphologies and functions but they are derived from the same precursor, the pluripotent stem cell. Under the influence of haematopoietic growth factors the stem cell generates the wide variety of haematopoietic cells. IL-3, IL-4, IL-6 and GM-CSF all act on early multipotential cells whereas G-CSF, M-CSF, IL-5 and erythropoietin act on later cells that are already committed to one or two lineages; this work is reviewed in Whetton & Dexter 1989,

Daniel & Dexter 1989. Figure 6 illustrates how these factors form a complex control system over the haematopoietic system.



Each haematopoietic growth factor has a unique, high-affinity receptor. These receptors are distributed in a manner that reflects their action (Nicola 1989) e.g., granulocyte-colony stimulating factor receptors are found on granulocytes. The discovery of a common 210 amino acid sequence in the extracellular domain of haematopoietic growth factor receptors, has led to the formation of a new hematopoietin receptor superfamily. The members of this family include: IL-2, IL-3, IL-4, IL-6, IL-7, GM-CSF, prolactin, growth hormone, and erythropoietin (Cosman *et al* 1990)

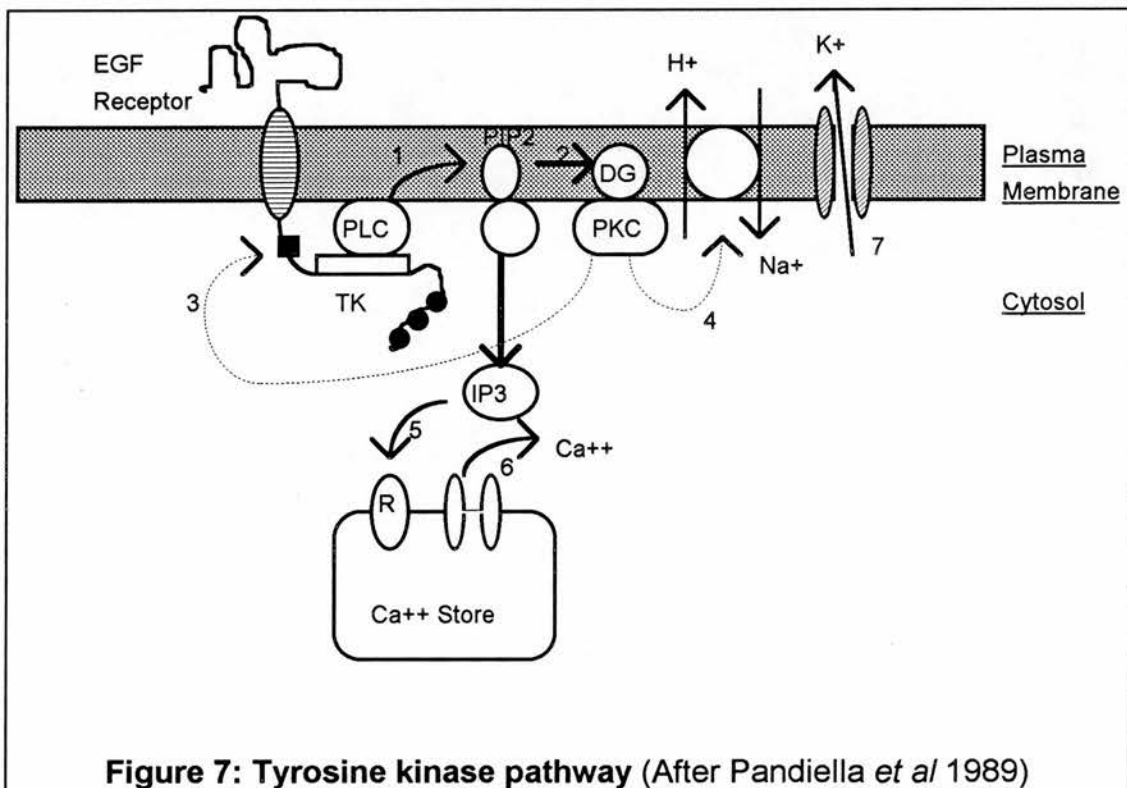
Several haematopoietic growth factors are used for cancer therapy. The ability of GM-CSF, G-CSF, M-CSF, IL-3 and IL-1 to influence all aspects of haematopoietic cell differentiation and proliferation has led to their clinical

use of reviving bone marrow growth after chemotherapy. They are also used for the treatment of myeloid leukaemia and other bone marrow disorders (Moore 1990). The ability of erythropoietin to stimulate erythropoiesis has led to its use in the treatment of anaemia (Adamson & Eschbach 1990, Oster *et al* 1990).

iii) Pathways of Growth Factor action

Each growth factor exerts its action through a specific receptor located in the cell membrane. The result of this binding is the activation of a variety of secondary messenger systems which ultimately influence cell metabolism. Two of the commonest systems are the tyrosine kinase pathway (figure 7) and the adenylate cyclase (figure 8) pathway. These are named according to the initial enzyme activated by each growth factor.

To give an indication of the complexity of growth factor - receptor signalling, the tyrosine kinase pathway is explained in more detail below.

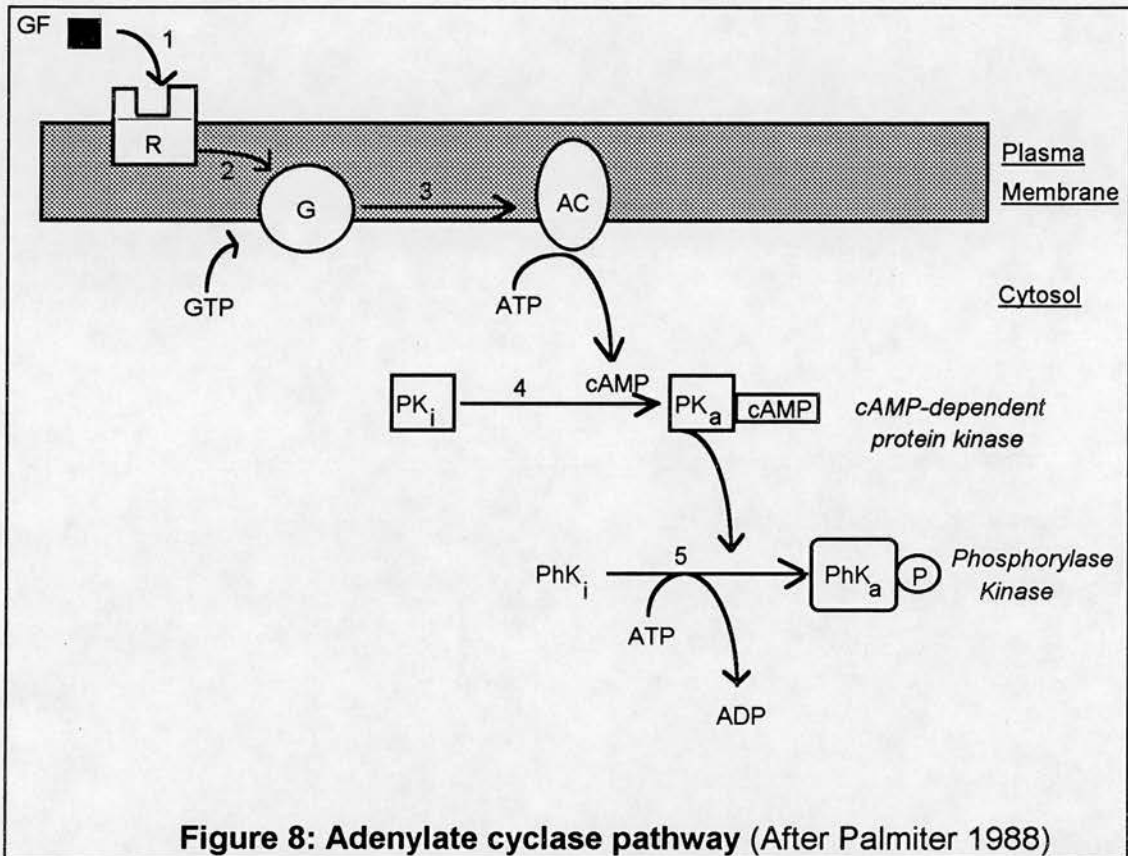


Most growth factors use the tyrosine kinase pathway; the example used here is EGF (figure 7). The binding of EGF to its receptor activates a tyrosine kinase (TK) in the cytoplasmic domain of the receptor. Evidence suggests tyrosine kinase (1) may in turn activate phospholipase C (PLC) by tyrosine phosphorylation (Meisenhelder *et al* 1989). This consequently leads to the hydrolysis (2) of phosphatidylinositol bisphosphate (PIP₂) to diacylglycerol (DG) and inositol triphosphate (IP₃). The first of these secondary messengers, diacylglycerol, activates protein kinase C (PKC). This active kinase can desensitise the EGF receptor (3), by phosphorylation of threonine 654 (Schlessinger 1988) and also activate (4) the sodium ion (Na⁺)/proton (H⁺) antiporter (Pouyssegur 1985). Meanwhile, inositol triphosphate bound to its receptor (R) on the surface of intracellular calcium stores (5) causes the release of calcium ions (Ca⁺⁺) (6). High levels of intracellular calcium ions activates a calcium dependent potassium (K⁺) channel (7) leading to hyperpolarization. The intracellular levels of calcium oscillate when the phosphoinositide pathway is stimulated. The frequency of these oscillations varies with agonist concentration (Berridge & Irvine 1989). Calcium signals can spread from cell to cell through gap junctions, enabling cell to cell communication.

The haematopoietin receptor family mediates tyrosine kinase activation via the extracellular domains of the receptor (Chiba *et al* 1993).

Many hormones and neurotransmitters use the adenylate cyclase pathway to produce cell proliferation, this is illustrated in figure 8. The binding of the hormone to its receptor (1) leads to the activation of a G protein (2). By a series of events adenylate cyclase (AC) can be inhibited or stimulated (3) to catalyse the production of cAMP from ATP. cAMP acts as a secondary messenger by activating a cAMP dependent protein kinase (4).

The activated kinase is then capable of phosphorylating a variety of other proteins in order to alter their function e.g., phosphorylase kinase activation (5).

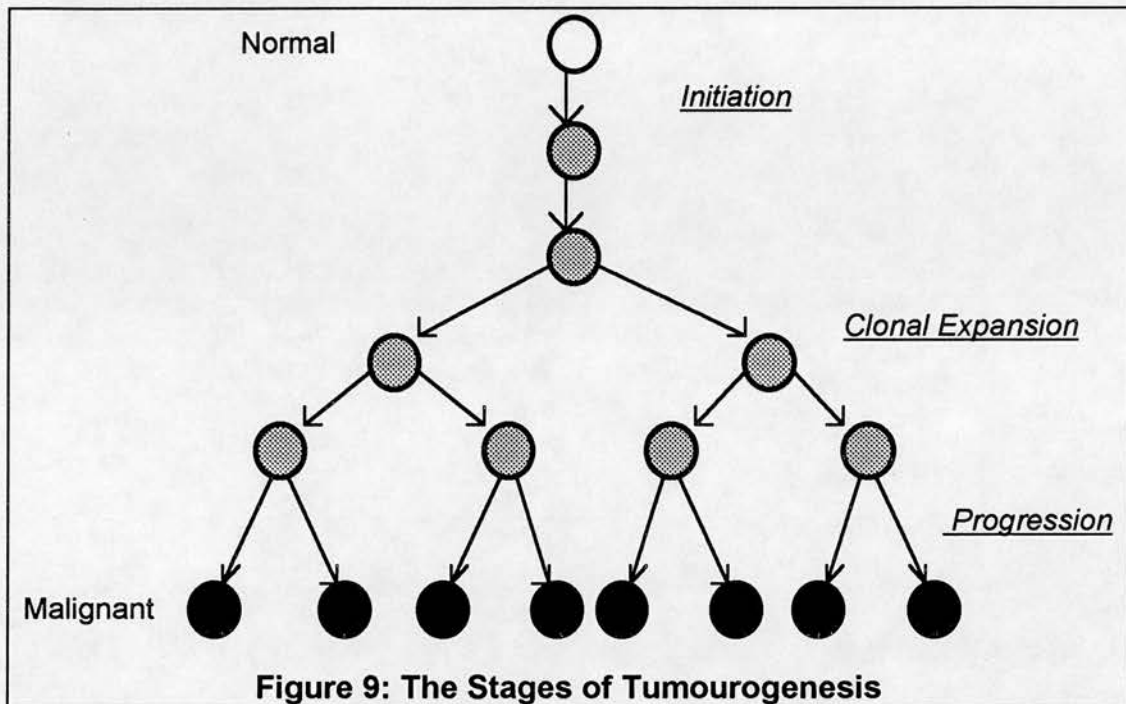


These pathways illustrate how growth factors form complex intracellular signals which control and co-ordinate cell metabolism and development.

The process is not infallible, and the mutation of a growth factor or any component of the intracellular signalling process can result in uncontrolled growth, and the development of a tumour. (Aaronson 1991). Such mutations are the result of oncogenes and tumour suppressor genes in the DNA sequences responsible for growth regulation (Bishop 1991, Cross & Dexter 1991).

c) Tumourogenesis & Genetic mutation

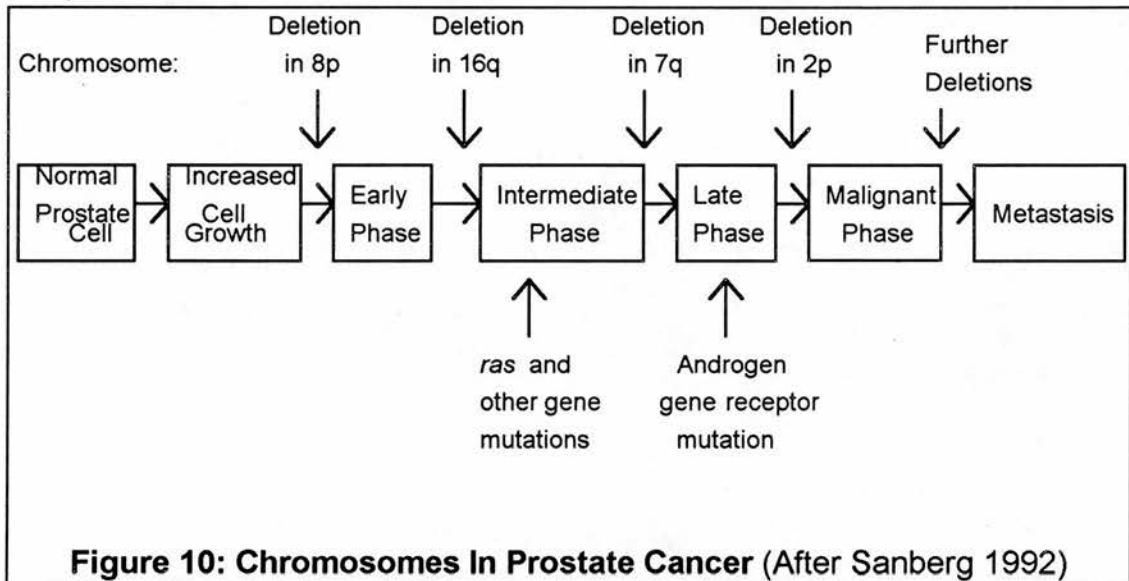
Tumourogenesis is thought to occur in a step wise fashion (figure 9):



The initial event of tumourogenesis is the mutation of genomic DNA. DNA can be mutated by the inheritance of a genetic defect, radiation, chemical carcinogen or viral alteration. This mutation, although inheritable, may have no effect on phenotype allowing it to remain dormant for years. However further mutation can cause aberrant protein expression and clonal expansion of the cell. The mutation can activate either an oncogene or deactivate a tumour suppressor gene (Bishop 1991). Additional genetic mutations cause the tumour to progress further and attain an autoregulatory state. Ultimately it becomes metastatic and invades other tissues.

Deletions are the most likely cause of tumour suppressor gene deactivation and these have been observed in prostate cell lines (Bookstein *et al* 1990, Brothman *et al* 1990, Carter *et al* 1990a). Oncogenes are dominantly acting mutant genes, whose presence produces a malignant

phenotype. Activation of an oncogene can occur through point mutation, chromosome translocations or gene amplification. Such mutations have also been identified in prostate cell lines and tumours (Nag and Smith 1989, Carter *et al* 1990b). Figure 10 illustrates how a hypothetical series of genetic mutations might accumulate and lead to the development of prostate carcinoma.



Investigations of prostate cancer cells have shown the presence of various mutations in the androgen receptor (Brinkmann *et al* 1991, Newmark *et al* 1992), and the total absence of the receptor in the hormone insensitive human prostate cell lines, DU145 and PC-3 (Tilley *et al* 1990). It is possible that both loss or mutation of the androgen receptor (Ekman *et al* 1991) and the attainment of aberrant growth factor action are involved in the progression of prostate cancer to a hormone insensitive state (King 1990, Davies & Eaton 1991).

d) Malignancy and growth factors

Mutations may cause overexpression of normal proteins or normal expression of mutated proteins, both resulting in the loss of normal growth control. There are several ways aberrant growth factor or action may aid tumourogenesis and these are discussed below:

i) Receptor mimicking

Sequence analysis of the avian *v-erb-B* oncogene product shows homology with the human EGF receptor sequence (Downward *et al* 1984). *v-erb-B* is thought to be a truncated form of the EGF receptor with intact membrane and cytoplasmic regions but no extracellular EGF binding domain. The lack of this binding domain may lead to a permanently activated receptor. Evidence also suggests that the *v-fms* oncogene may represent a mutated M-CSF receptor. The receptor still retains an M-CSF binding site but a deletion has led to the loss of the tyrosine autophosphorylation site important for desensitisation. As a result the receptor is constitutively activated (Coussens *et al* 1986), and known to cause fibrosarcomas. Transcripts for the *fms* oncogene have been identified in ovarian adenocarcinomas (Kacinski *et al* 1990), breast carcinomas (Kacinski *et al* 1991) and lung carcinoma cell lines (Filderman *et al* 1992). In all cases expression was thought to be connected to invasiveness.

ii) Autocrine stimulation

The production of a growth factor by a cell which is stimulatory for itself is known as autocrine stimulation. In malignancy this can lead to the loss of dependency from paracrine or endocrine sources of normal growth stimulation and the cell grows uncontrolled (Sporn & Roberts 1985). Research has shown that prostate carcinoma cells can produce and respond to $TGF\alpha$ (Wilding *et al* 1989a, MacDonald *et al* 1990) and $TGF\beta 1$ (Steiner &

Barrack 1992) in an autocrine fashion. The secretion of GM-CSF by Lewis-lung carcinoma cells was also implicated in autologous stimulation of these cells since they also proliferate in response to exogenous GM-CSF (Young *et al* 1992). Furthermore the ability of these cells to invade basement membrane was inhibited by a GM-CSF antibody indicating a possible role in the metastatic properties of these cells.

iii) Paracrine Influences and metastasis

Growth factors released by one cell type may increase the tumourogenic potential of another. For example, androgens are known to stimulate hormone sensitive prostate tumours (Schuurmans *et al* 1991). Certain growth factors such as GM-CSF have been implicated in developing a cell's invasive potential (Young *et al* 1992). GM-CSF gene expression has been correlated with metastatic tumour formation in mice (Takeda *et al* 1991), tumour necrosis factor was found to increase the metastatic potential of Chinese hamster ovary cells (Malik *et al* 1990), and the presence of M-CSF receptors on lung carcinoma cells is linked to their metastatic ability (Filderman *et al* 1992).

Paracrine influences are important for metastasis. A paracrine growth factor can also act as a motility factor (Gherardi 1991); or have angiogenic properties allowing the tumour to increase in size (Bicknell & Harris 1991); or they may stimulate tumour cells within the secondary metastatic site. These factors are discussed further in the next section.

1.3 Metastasis

Metastasis describes the spread of cancer cells from the primary malignant tumour to secondary sites in other organs. The tumour cell must complete all the steps of the metastatic cascade (illustrated in figure 11) to form a secondary tumour.

With the number of 'obstacles' to overcome it can be seen why metastasis has been described as an inefficient process (Weiss 1990). This was illustrated by Graves (1983) who found that although thousands of malignant cells may enter the blood stream only a small percentage will form secondary tumours. Despite this inefficiency, metastasis occurs in many cancer patients, and is invariably the cause of death.

Observations have indicated that metastases originating from a primary tumour have a tendency to form in specific target organs, examples of this can be seen in table 4.

Table 4: Common metastatic sites of cancer tissues (After Hill 1987)

PRIMARY TUMOUR	COMMON METASTATIC SITE
Kidney carcinoma	lung, bone, adrenal
Prostate carcinoma	bone
Small cell lung carcinoma	brain, liver, bone marrow
Melanoma of the skin	liver, brain, bowel
Melanoma of the eye	liver
Neuroblastoma	liver, adrenal
Thyroid, follicular carcinoma	bone, lung
Breast carcinoma	bone, brain, adrenal, lung

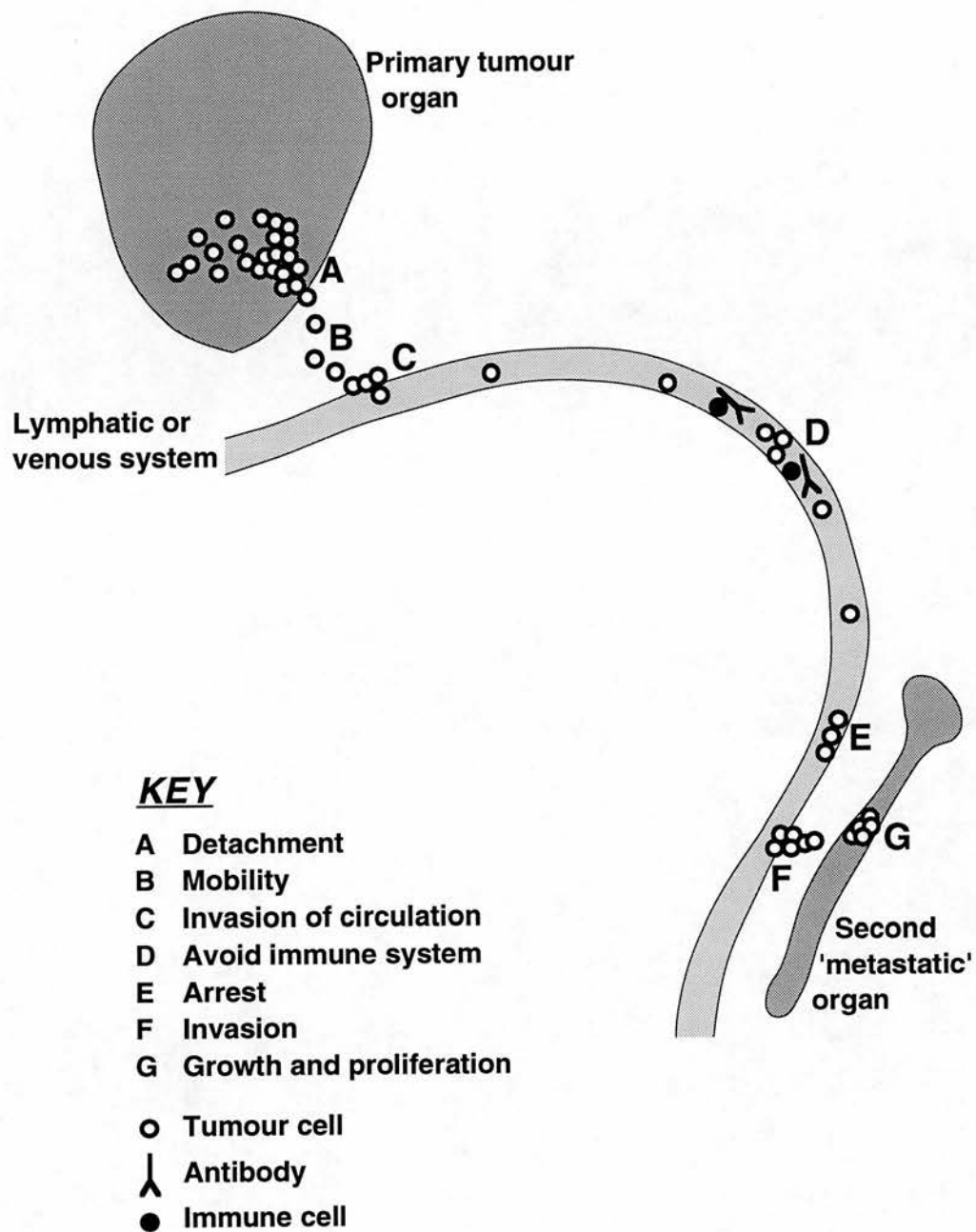


Figure 11: The Metastatic Cascade

The observation of cancers metastasising to specific secondary sites supports the idea of Stephen Paget's 'Seed and Soil' hypothesis (Paget 1889). The hypothesis suggests that certain organs may provide a suitable environment of growth factors (soil) for the growth of metastatic tumour cells (seed). However it is likely that a number of factors are involved in the formation of site specific metastases. These are now discussed by studying the steps of metastatic cascade in detail, with particular reference to prostate carcinoma.

a) The Metastatic Cascade

i) Detachment From The Primary Tumour

A metastatic cell must initially escape the primary tumour. This involves the loss of cell to cell contact which may occur through a decrease in the levels of adhesion molecules. These ideas are strengthened by results indicating that fibronectin mRNA is lower in metastatic rat prostate cell lines in comparison to equivalent non-metastatic lines (Schalken *et al* 1988). Also the expression of E-cadherin (a calcium dependent cell adhesion molecule) decreases with the increasing invasive potential of a series of rat prostate cell lines (Bussemakers *et al* 1992).

ii) Mobility

In order to reach the circulation a metastatic cell must become mobile. Nerve growth factor-like proteins secreted from prostate stroma have shown the ability to stimulate prostate cancer cell line motility (Djakiew *et al* 1993). Autocrine motility factors have been found, secreted from melanoma cells (Liotta *et al* 1986). Degradation products of the basement membrane can also induce cell mobility (Blood & Zetter 1990). These include molecules such as fibronectin, collagen and laminin (Lacovara *et al* 1984, Mundy *et al*

1981, Wewer *et al* 1987). Hydrolytic enzymes secreted by invading tumour cells are responsible for the degradation of extracellular matrices.

iii) Invasion Of The Circulation

Research has led to the discovery of a variety of enzymes, secreted by tumour cells, that are capable of degrading basement membrane. The release of such enzymes allows the invasion of either the venous system or the lymphatics. One such enzyme is plasminogen activator, it exists in two forms: urokinase type (u-PA) and tissue type (t-PA). Plasminogen activator catalyses the activation of plasminogen to plasmin. Plasmin is an enzyme capable of degrading many proteins including laminin and fibronectin (Liotta *et al* 1981). It may also activate latent collagenase (Salo *et al* 1982), therefore indirectly degrading collagen.

The central zone of the prostate is a selective site for t-PA in seminal fluid (Reese *et al* 1988). Elevated levels of plasminogen activator have been found in cancerous prostate tissue. Results have shown malignant prostate tissue extracts have higher u-PA activity than benign, and bone metastases have higher plasminogen activator levels than the primary tumour (Kirschheimer & Binder 1990). Plasminogen activator content has also been linked to metastatic potential of prostate cancer cell lines LNCaP, DU145 and PC-3 (listed in order of increasing aggressiveness). Keer *et al* (1991) demonstrated PC-3 cells have twice the activity of u-PA than DU145 cells. DU145 and PC-3 cells both secrete and have receptors for u-PA, whereas LNCaP has neither characteristic (Hoosein *et al* 1991). Furthermore the invasive properties of DU145 and PC-3 can be inhibited using an urokinase receptor agonist.

A second family of enzymes, the metalloproteinases, have also been linked to invasion. Human matrix metalloproteinase-7 degrades gelatins,

fibronectin and activates collagenases. Using Northern blots matrix metalloproteinase-7 transcripts were found three times more frequently in prostate adenocarcinomas than normal tissue samples. No evidence for matrix metalloproteinase-7 transcripts in prostate cell lines were found (Pajouh *et al* 1991).

These results provide good evidence for the involvement of proteolytic enzymes in the metastasis of prostate cancer.

iv) Avoidance Of The Immune System

Once in the circulation a tumour cell must survive the immune system. Avoidance of the body's natural defences may be achieved through the alteration of MHC antigens - consequently the tumour cells are not recognised by T-lymphocytes (Katzav *et al* 1984). Tumour cells are known to aggregate platelets, but how this aids metastasis is unknown. Potentially, the platelets form an embolus around the tumour cells, thereby masking them from the immune system (Gasic 1984). These emboli may also be important for arresting at secondary sites.

v) Arrest At A Secondary Site

Tumour cells arrest at the vascular endothelium of secondary metastatic sites either through specific receptor interactions or adhesion molecules, or simply where the cells become physically lodged (Johnson *et al* 1991, Blood & Zetter 1990). Adhesion molecules such as laminin and fibronectin (Terranova *et al* 1984, Haberern *et al* 1985) are thought to be major determinants of site specific metastases (Zetter 1990). Evidence demonstrates that tumour cells will preferentially adhere to the extracellular matrix components of the organ they metastasise to (Doerr *et al* 1989). Rat prostate adenocarcinoma cell lines have shown preferential adherence to

bone marrow endothelial cells over bone marrow stromal cells, osteoblasts, hepatic endothelial cells or rat fibroblasts (Haq *et al* 1992).

Once the tumour cell has bound to the endothelial surface, cell retraction occurs exposing the subendothelial basement membrane.

vi) Invasion Of Secondary Organ

The basement membrane is again a barrier to the passage of metastasising cells from the circulation into the secondary tumour. The variety of hydrolytic enzymes originally required to escape the primary tumour probably participate again. However the presence or absence of a particular enzyme may correlate with a tumour cells ability to invade a specific tissue (Zetter 1990), since the components of the extracellular matrix differ from tissue to tissue. Experiments have also shown the organ environment can affect the type of degradative activity produced by the tumour cell (Nakajima *et al* 1990). Site specific metastasis is therefore also governed by the type of hydrolytic enzyme a tumour produces.

vii) Growth and Proliferation At The Metastatic Site

Once at the metastatic site, tumour cells must grow and proliferate even though this is a foreign environment. Growth factors secreted from the secondary organ are thought to be important in maintaining growth. The presence of distinct growth factors in different secondary organs may also lead to site specific metastases (Nicolson *et al* 1986). Prostate cancer shows a selective metastatic spread to bone (Jacobs 1983), therefore growth factors released by the skeleton may aid the establishment and proliferation of a prostate skeletal secondary tumour.

1.4 Bone

Bone is a highly specialised connective tissue with organic and inorganic components, that are continually remodelled.

a) Normal Structure and Function

Bone serves as a support for soft tissue and protects vital organs, e.g., the skull protects and supports the brain. Other functions involve the small bones of the ear for sound transmission, and the calcified skeleton which acts as a reservoir for ionic calcium. There are two types of bone, compact and cancellous. Compact bone is very dense and is also referred to as cortical bone. It forms the major component of long bones providing structural support. Microscopic cylindrical structures known as haversian systems are an integral part of this bone. They are made of concentric collagen lamellae, surrounding nutrient blood vessels. Cancellous bone is also known as spongy or trabecular bone. It is composed of networks of interconnecting plates (trabeculae) surrounded by bone marrow. The composition makes it less dense than compact bone, though more biologically active; for reviews see Boyce 1991, Williams & Frolik 1991.

b) Normal Growth

Since bone must endure much stress and strain, it is prone to wear and tear and therefore constant remodelling is essential to prevent weakening. There are three main cell types present in bone, which are involved in the remodelling process:

i) The Osteoclast

This cell is multinucleate and responsible for bone resorption. It is generally accepted to derive from the macrophage-monocyte lineage in the bone marrow (Kurihara *et al* 1990). The osteoclast is polar and has a brush border in close contact with the bone surface forming an enclosed extracellular space. Into this space it actively secretes degradative enzymes (Delaisse *et al* 1987) and creates an acid environment (Baron *et al* 1985). The enzymes are activated by the low pH enabling solubilization of the bone matrix (Russel *et al* 1990).

ii) The Osteoblast

Osteoblasts are mononuclear and responsible for bone formation. Evidence suggests they originate from stromal cells in the bone marrow (Vaughan 1981). Osteoblasts produce an organic matrix known as osteoid which is essential for mineralization. Collagen type I is the main component of osteoid, though the osteoblast secretes many more factors, listed in tables 5 and 6.

Table 5: Components of Osteoblast Extracellular Matrix

Collagen I	(Owen <i>et al</i> 1990)
Fibronectin	(Owen <i>et al</i> 1990)
Osteonectin	(Russel <i>et al</i> 1990)
Osteocalcin	(Aronow <i>et al</i> 1990)
Chondroitin sulphate	(Fedarko <i>et al</i> 1990, Takeuchi <i>et al</i> 1990)
Heparan sulphate	(Fedarko <i>et al</i> 1990, Takeuchi <i>et al</i> 1990)
Hyaluronan	(Fedarko <i>et al</i> 1990)
Dematan sulphate	(Fedarko <i>et al</i> 1990, Takeuchi <i>et al</i> 1990)

Table 6: Growth Factors Released By Osteoblasts

TGF α	6Kd	(Cantrella & Canalis 1987)
TGF β	25Kd	(Robey et al 1987)
TNF α	17Kd	(Gowen et al 1990)
IGF-I	7Kd	(Slootweg et al 1990)
IGF-II	7Kd	(Slootweg et al 1990)
GM-CSF	15-22Kd	(Horowitz et al 1989)
M-CSF	14-21Kd	(Felix et al 1989)
G-CSF	19-24Kd	(Felix et al 1991)
IL-1	17Kd	(Hanazawa et al 1987)
PDGF-like peptides	24-34Kd	(Valentin-Opran et al 1987)
Alkaline phosphatase		(Aronow et al 1990)
Plasminogen activator	54-70Kd	(Pfeilschifter et al 1990)
Plasminogen activator inhibitor		(Pfeilschifter et al 1990)
9K bone resorbing protein		(Morris et al 90)

Populations enriched in osteoblasts can be cultured using sequential digestion procedures (Wong & Cohn 1974). This relies on the fact that osteoblasts are present in distinct areas of the bone. Osteoblasts have also been cultured as outgrowths from small chips of trabecular bone (Beresford et al 1984).

iii) The Osteocyte

This cell is formed from the osteoblast and is the most numerous of the three cell types. It is in effect the osteoblast left behind, trapped, in its own calcified matrix. It has no function in bone formation, but networks of these cells, joined by long dendrites, maybe involved in the bone's response to stress (Russel et al 1990).

c) Remodelling

The remodelling process is controlled by three main hormones: parathyroid hormone, calcitonin and Vitamin D. Parathyroid hormone is

secreted in response to decreased calcium and leads to bone resorption, whereas calcitonin is secreted (from the thyroid) in response to increased calcium levels and inhibits resorption (Russel *et al* 1990). Vitamin D increases calcium levels by stimulating resorption (Russel *et al* 1990). Table 7 lists the variety of other growth factors, local or humoral which are involved in the remodelling of bone.

Table 7: Factors Affecting Bone remodelling

Autocrine/paracrine Growth Factor	Bone Formation	Resorption	Ref
EGF	Inhibition	Stimulation	1,2
aFGF, bFGF	Osteoblast mitogen		3
IGF-I	Bone cell mitogen		4
IGF-II	Bone cell mitogen		5
IFN γ		Inhibition	6
TGF β	Potent stimulator	Inhibition	7
PDGF	Bone cell mitogen		8
TNF α		Stimulation	9
IL-1		Stimulation	10
GM-CSF	Stimulation	Stimulation	11,12
HEP I & II Proteins	Osteoblast mitogen		13
Parathyroid hormone related peptide		Stimulation	14
Prostaglandin E2		Stimulation	15,16
Bone morphogenic proteins	Stimulation		17
Endocrine Factor			
Calcitonin		Inhibition	18,20
Parathyroid hormone		Stimulation	19,20
1,25 dihydroxy vitamin D		Stimulation	12
Glucocorticoids		Stimulation	21
Growth hormone	Osteoblast mitogen		22
Insulin	Stimulation		23
Oestrogen	Stimulation		24

References: 1) Canalis *et al* 1979, 2) Simpson 1984, 3) Rodan *et al* 1987, 4) Canalis 1980, 5) Mohan & Baylink 1991, 6) Peterlik *et al* 1985, 7) Robey *et al* 1987, 8) Canalis 1981, 9) Bertolini *et al* 1986, 10) Gowen *et al* 1983, 11) Evans *et al* 1989, 12) Kurihara *et al* 1990, 13) Rifas *et al* 1989, 14) Morris *et al* 1990, 15) Pfeilschifter *et al* 1990, 16) Peterlik *et al* 1985, 17) Urist *et al* 1984, 18) Chambers & Magnus 1982, 19) Raisz *et al* 1965, 20) Heath & Reynolds 1990, 21) Russel *et al* 1990, 22) Sloatweg *et al* 1988, 23) Canalis *et al* 1977, 24) Sloatweg *et al* 1992

The remodelling process is continual. The recruitment of osteoclasts by both vitamin D and PTH leads to the release of hydrolytic enzymes.

'Resorption bays' are formed at local sites of bone degradation. By unknown methods, resorption is coupled to the migration and proliferation of osteoblasts (Russel *et al* 1990). Osteoblasts lay down the osteoid matrix which is subsequently mineralised by deposition of hydroxyapatite to produce new bone (Owen *et al* 1990).

The osteoblast and osteoclast are coupled to the extent that often the receptors for factors causing resorption are found on the osteoblast e.g., parathyroid hormone receptors are localised to the osteoblast (Newman *et al* 1989). The action of parathyroid hormone is thought to release soluble growth factors from the osteoblast which then stimulate osteoclastic resorption (McSheehy & Chambers 1986). This indicates how complex mechanisms are likely to exist between the two cells. Various candidates exist for a coupling factor between osteoblasts and osteoclasts, including TGF- β (Bonewald & Mundy 1990), parathyroid hormone-related protein (Morris *et al* 1990), GM-CSF (Evans *et al* 1989) and HEP (Rifas *et al* 1989) proteins.

d) Skeletal Metastasis

Skeletal metastases are known to be common in patients dying from advanced cancers of the prostate, breast, bronchus, kidneys and thyroid (Galasko 1986), whereas cancers of the alimentary canal rarely produce such metastases. Pain is the main symptom for the presence of a skeletal metastasis. They are more often found in cancellous bone than cortical, probably due to differences in biological activity (as detailed before).

Malignant cells can either produce an osteoblastic or osteolytic effect on the skeleton (Boyce 1991).

An osteolytic metastasis causes a net destruction of bone, leading to its weakening. The malignant cells of an osteolytic metastasis are thought to secrete osteoclast activating factors, therefore bone destruction is indirect (Galasko 1976, Mundy 1988). Studies have in fact shown that malignant cells can secrete factors known to stimulate osteoclastic activity, these include cytokines and growth factors such as $TGF\alpha$, $TGF\beta$ and EGF (Tashjian *et al* 1985).

In an osteoblastic metastasis the malignant cells cause a proliferation of osteoblasts and therefore increased bone mass. This most likely occurs due to the production of osteoblast growth factors by the malignant cell (Simpson *et al* 1985), but may also involve mechanisms separate from normal remodelling (subsequent resorption is not seen) or a loss of the coupling mechanism between the osteoblast and the osteoclast (Boyce 1991).

e) Prostate Skeletal Metastases

As has been stated, prostate cancer shows a selective metastatic spread to bone. The most common sites of prostate skeletal metastases are the spine, femur, pelvis, ribs, sternum, skull and humerus (Jacobs 1983). These metastases are osteoblastic, though both bone formation and degradation are seen.

Metastases appear first in the bone marrow, where they can survive undetected for a long time. This dormant stage maybe due to angiogenesis, or the host destroying cancer cells as fast as they divide. Eventually growth of the tumour cells becomes dominant. Once established the cancer expands, invades and remodels the surrounding bone. The tumour cells must somehow activate osteoclasts to allow initial degradation. Galasko

(1976) and Tashjian *et al* (1985) both produced results suggesting tumour cells can produce osteoclast activating factors. After invasion, the cancer produces a proliferation of osteoblasts, accompanied by new bone formation (Jacobs 1983), leading to irregular and broader remodelling. The proliferation of osteoblasts may be due to growth factors released by the tumour. These factors may cause osteoblastic recruitment or affect their production of osteoid.

There are at least four reasons (which are not exclusive) why prostate cancer metastasises preferentially to the bone. The first is mechanical. Batson (1940) suggested metastasis to a specific site depends on the specific vasculature the cancer cell invades. He demonstrated prostate cancer cells would invade the vertebral venous system, (a network of longitudinal, valveless veins which run parallel to the vertebral column) and form large anastomoses with the vertebrae, pelvis, thorax and brain venous systems. Therefore this venous network could account for the pattern of secondary skeletal tumours. Secondly, specific adhesion molecules in the bone marrow maybe important for the arrest of the tumour cell. Thirdly, any degradative enzymes secreted by the prostate cancer cells which allow the remodelling of the surrounding bone, maybe important. Lastly, Stephen Paget (1889) hypothesised cancers seeded at secondary sites only where the environment was favourable for growth, due to the presence of stimulatory growth factors. Since prostate tumour cell growth is greater in the bone environment than in the primary tumour (Jacobs 1983, Berretoni *et al* 1986) this would suggest skeletal growth factors do stimulate prostate cancer cell growth.

It becomes clear that factors from the bone environment and the tumour cells are acting very closely, potentially in a paracrine manner. This is now discussed further.

i) Growth Factors Involved In Prostate Skeletal Metastases

Bone Growth Factors Produced By Prostate Cancer Cells

Many researchers have looked for a prostatic growth factor that is capable of producing osteoblastic metastases. There are many growth factors known to be secreted by the prostate (see table 2), some of these can stimulate the osteoblast (table7).

In 1979 Jacobs *et al* were the first to show that extracts of human benign prostatic hyperplasia contained a mitogen for rat osteoblast cells. A second research group used reverse phase high performance liquid chromatography on extracts of both benign prostatic hyperplasia and prostate carcinoma tissue, and found a peptide of 10 Kd that had selective mitogenic activity on rat osteoblast cells (Koutsilieris *et al* 1987). A rat osteoblast cell line was again stimulated by media conditioned by the prostate cell line, PC-3 (Harrod *et al* 1985), this stimulatory activity was isolated as a factor of 20Kd. PC-3 cell mRNA was purified and injected into *Xenopus laevis* oocytes by Simpson *et al* (85). This led to the formation of new translation products from the oocytes which were secreted into the culture medium, this conditioned medium (CM) had mitogenic activity on rat osteosarcoma cell lines. The CM was also capable of increasing a number of bone formation associated activities (collagen synthesis and alkaline phosphatase activity). The majority of this activity was shown to correspond to a mRNA fraction of 1800 base pairs, which could encode the 20Kd product.

Perkel *et al* (90) were the first to use primary cultures of human osteoblast-like cells. Their experiments demonstrated that PC-3 CM could stimulate these cells but not human skin fibroblasts. Known growth factors (TGF β , IGF I, IGF II) were also used to stimulate the osteoblast-like cells with and without the CM. The CM had a cumulative effect with these factors suggesting the stimulatory factor in the CM was not one of the common factors tested and chromatography indicated its molecular weight to be in the order of 26-30Kd.

Bone Growth Factors Acting On Prostate Cancer Cells

It is a common occurrence that prostate cancer cells grow at a faster rate in skeletal metastases than in primary tumours (Jacobs 1983, Berrettoni *et al* 1986). Several researchers have looked for growth factors released by the bone environment that are mitogenic for prostate cancer cells.

Charkal-Roy *et al* (1989) were able to stimulate the growth of the cell lines PC-3 and DU145 using media conditioned by fresh human bone marrow (BMCM) and by primary cultures of human bone marrow stromal cells. These CM had little effect on cell lines derived from other metastatic tumours. The prostate cell lines were also incubated with haematopoietic growth factors to determine if the BMCM stimulation was due to haematopoietic growth factors in the bone marrow. Only M-CSF was seen to produce a 100% stimulation of growth but this did not match the 800% stimulation from the BMCM, and no combination of haematopoietic growth factors could achieve this. Later studies showed that transferrin was one of the stimulatory components of BMCM (Charkal-Rossi *et al* 1992).

Charkal-Roy's results are in contrast to earlier results by Klose *et al* (1987) who demonstrated that rat bone marrow contained a 640 Dalton factor which inhibited a rat prostate cancer cell line and the human cell line

DU145. The finding of a rat cell line inhibitory factor in bone marrow may help explain the lack of skeletal metastases in the Dunning rat model of prostate carcinoma. The result for the DU145 cells is less clearly explained unless the inhibition was due to a cross-species effect.

Further studies by Rago *et al* (1991) are also contrary to Charkal-Roy's results, Rago showed bone marrow stromal cell CM had no growth effects on either PC-3 or DU145 cells and inhibited the growth of the hormone sensitive cell line, LNCaP. Whether this result was due to androgen sensitivity or not, (DU145 and PC-3 cells are androgen insensitive), was not determined. The experiments involved a filtration step (5000 molecular weight cut off) which could have removed Charkal Roy's stimulatory factor.

Gleave *et al* (1991) coinoculated athymic nude mice with LNCaP cells and various non-tumorigenic fibroblasts. The results indicated that a human bone fibroblast cell line (MS) was the most effective at producing LNCaP tumours, (followed by rat prostate fibroblasts and then embryonic rat urogenital fibroblasts). The results were strengthened by the fact bone fibroblast CM stimulated LNCaP growth 150% in comparison to a control, *in vitro*. However CM from prostate fibroblasts and also bFGF produced similar stimulatory effects. These results indicated the presence of a growth factor from bone fibroblasts that stimulate LNCaP cell line growth.

As a whole, the results from all these experiments are rather confusing, since Charkal-Roy *et al* (1989) demonstrated the stimulation of prostate cell line growth by BMCM, despite contradictory experiments by Klose *et al* (1987) and Rago *et al* (1991). Although Gleave *et al* (1991) showed stimulation of LNCaP cells by bone fibroblasts this wasn't specific to the bone and the experiment was carried out using a mouse model.

Much evidence exists for paracrine growth factors mediating stimulatory activity between the bone environment and prostate cancer. Such factors could also be responsible for the selective metastasis of prostate carcinoma to the skeleton. No-one has yet looked specifically for an osteoblast-derived factor that can stimulate prostate cancer growth.

Another way of investigating the site specific metastasis of prostate cancer to bone would be to test haematopoietic growth factors for growth stimulation, since they are produced largely in the bone marrow (Nicola 1989). Although a number of researchers (EORTC 1991, Twentyman & Wright 1991) demonstrated no growth stimulation of human carcinoma cell lines by haematopoietic growth factors, other groups have shown stimulatory actions of these factors on non-haematopoietic cell lines (Table 8).

Table 8: Stimulatory activity of haematopoietic growth factors on non-haematopoietic cell lines

Haematopoietic Growth Factor	Cell Line Stimulated
GM-CSF	Lung (Young et al 1992, Nakata et al 1991)
	Bladder (Block et al 1992)
	Breast (Dedhar et al 1988)
	Colorectal (Berdal et al 1992)
IL-3	Bladder (Block et al 1992)
	Colorectal (Berdal et al 1992)

The effect of haematopoietic growth factors on prostate cell line growth has not been extensively investigated.

Objectives

Prostate cancer has a preferred metastatic spread to bone. A number of events are thought to be important in the formation of a site specific metastasis: a) mechanical i.e., vertebral venous spread; b) selective adhesion at secondary sites due to the presence of specific adhesion molecules; c) tumour production of hydrolytic enzymes capable of degrading the secondary organ's extracellular matrix; d) the ability of a secondary organ to sustain and increase growth of a tumour cell depending on the growth factor it produces.

This study aims to investigate the latter step by examining the influence of:

- a) human osteoblast conditioned media
 - b) human bone marrow conditioned media
 - c) recombinant haematopoietic growth factors
- on human prostate cell line growth.

Also to determine if prostate cell lines produce any of the haematopoietic growth factors.

CHAPTER 2

METHODS & MATERIALS

2.1 Materials

a) General Chemicals

Most general chemicals were purchased from either Fisons (Loughborough, UK) or Sigma Chemicals (Poole, UK). Chemicals purchased elsewhere are listed below:

Aldrich (Gillingham, UK): Hydrogen peroxide, isopropanol, sodium hydroxide pellets,

BDH (Poole, UK): DePeX mounting medium, sodium dihydrogen orthophosphate, tetramethylethylenediamine (TEMED).

Bio-Rad (Hemel Hempstead, UK): Bradford dye reagent concentrate.

James Buroughs (London, UK): Ethanol

MacKay & Lynn Distributors (Edinburgh, UK): Oxoid Dulbecco's A phosphate tablets (one tablet dissolved in 100ml of distilled water)

Spectrum (Pierce Warner, Chester, UK): Spectrapore 3 dialysis tubing (3.5Kd MW cut-off)

b) Tissue Culture

i) Cell Line Sources

Prostate carcinoma cell lines, previously discussed in the introduction, were obtained from the sources listed in table 1. All other human cell lines were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). Their names and derivations are listed in table 2.

Table 1: Prostate adenocarcinoma Cell Line Sources

Name	Description		Source
DU145	brain metastasis	hormone insensitive	D.Mickey, Dept. Urology, University N. Carolina, USA
PC-3	bone metastasis	hormone insensitive	European Collection of Animal Cell Cultures, Porton Down, UK
LNCaP	lymph node metastasis	hormone sensitive	C.Eaton, Tenovus Institute, Cardiff, UK

Table 2: Human Cell Lines

Name	Derivation
MG63	male osteosarcoma
RT112	epithelial bladder carcinoma
HF19	female, foetal lung fibroblast
Hs578T	epithelial breast carcinosarcoma
CHANG	liver epithelia
ACHN	male, renal adenocarcinoma, fibroblast
HEP 2	epithelial larynx carcinoma

ii) Disposables:

All the tissue culture disposables (Corning and Sterilin) were purchased from MacKay & Lynn Distributors (Edinburgh, UK). Centricon concentrators; -3 (MW cut-off 3Kd), -10 (MW cut-off 10Kd), -30 (MW cut-off 30Kd), -100 (MW cut-off 100Kd) were purchased from Amicon (Stonehouse, UK). Scintillation vials were purchased from Canberra Packard (Pangbourne, UK).

iii) Tissue Culture Media

Tissue culture media were purchased as follows:

Flow (High Wycombe, UK): Ham's F12 Medium

Gibco (Paisley, UK): Dmem/F12 Medium, foetal calf serum (FCS), L-glutamine (200mM), heat inactivated foetal calf serum (HIFCS), 10,000IU/ml penicillin/ 10,000µg/ml streptomycin solution, RPMI 1640 Medium, trypsin-EDTA (10x) solution.

Kyokutō Pharmaceuticals (Tokyo, Japan): WAJC 404 tissue culture medium

iv) Chemicals

Tissue culture grade chemicals were purchased from Sigma (Poole, UK) or from the sources listed below:

Amersham (Slough, UK): [methyl-3H] thymidine (25Ci/mmol).

Canberra Packard (Pangbourne, UK): 'Ultima Gold' scintillation fluid.

Lorne Diagnostics (Bury St.Edmunds, UK): Worthington collagenase No.4196 (SA>170U/ml)

Roche (Welwyn Garden City, UK): 1 α , 25-dihydroxycholecalciferol (1.25 (OH)₂ Vitamin D). This was dissolved to 10µM in ethanol and stored under nitrogen at -20°C.

c) Growth factors

The following haematopoietic growth factors were all human recombinant growth factors produced from *E.Coli*; erythropoietin (125mU/µg), interleukin-3 (1000U/µg), granulocyte colony stimulating factor (100,000U/µg), granulocyte macrophage-colony stimulating factor (10,000IU/µg). All were kind gifts from the National Institute For Biological Standards and Controls (Potters Bar, UK).

Natural mouse, epidermal growth factor was purchased from Collaborative research (c/o Universal biologicals, London, UK) and human platelet, transforming growth factor β 1 from British Biotechnology (Cowley, UK).

Transforming growth factor α (rat) was kindly donated by Dr. H.Gregory (ICI, Macclesfield, UK).

d) Antibodies

The monoclonal mouse anti-human GM-CSF (IgG₁) was purchased from Genzyme (West Malling, UK) and a second GM-CSF antibody (polyclonal sheep anti-human GM-CSF serum) was provided by the National Institute For Biological Standards and Controls (Potters Bar, UK). The human GM-CSF immunoassay, 'Quantikine Kit' was aquired from British Biotechnology (Cowley, UK).

Sheep serum and monoclonal mouse anti-human α fetoprotein (IgG₁ kappa) were both provided by SAPU (Carlisle, UK).

Anti-mouse Ig-POD, Fab fragments and donkey anti-sheep IgG peroxidase conjugate were purchased from Boehringer Mannheim Biochemica (Germany) and Sigma (Poole, UK), respectively.

e) Western Blotting

Materials and electrophoresis grade chemicals were purchased from the sources listed below:

Amersham (Slough, UK): ECL Western blotting analysis system, Hi-bond C extra nitrocellulose membrane, Hyperfilm ECL, Rainbow protein molecular weight markers 2.35-46-Kd and 14.3-200Kd.

Sigma Electrophoresis Grade Chemicals (Poole, UK): 40% acrylamide solution, ammonium persulfate, glycine, silver nitrate, tris base.

Strattech Scientific Ltd. (Luton, UK): 'Blue Juice' sample buffer (bromophenol blue, urea, SDS, mercaptoethanol).

f) Molecular Biology

i) Disposables

Sterile pastettes were purchased from Alpha Labs. (East Leigh, UK) and sterile 500µl micro-test tubes from Bio-Rad (Hemel Hempstead, UK). Elkay 1.5ml microcentrifuge tube (eppendorfs) and Elkay 50ml and 15ml sterile falcon tubes were all bought from MacKay & Lynn Distributors (Edinburgh, UK).

ii) Chemicals

General molecular biology grade chemicals were obtained from Sigma (Poole, UK). Chemicals and enzymes that were purchased elsewhere are listed below:

Appligene (Durham, UK): Aquaphenol (water saturated phenol)

Northumbria Biologicals (Cramlington, UK): Guanidine isothiocyanate

Pharmacia (Milton Keynes, UK): Agarose NA, 100 base pair ladder (reconstituted in 800µl autoclaved water+100µl loading buffer), ultrapure solution dNTP (100mM).

Promega (Southampton, UK): AMV reverse transcriptase (25 000U/ml), 5x AMV RT buffer (250mM Tris-HCl, pH 8.3, 250mM KCl, 50mM DTT, 2.5mM spermidine), *Bgl* I (20U/µl), 10x enzyme buffer (60mM Tris-HCl, pH 7.9, 1.5M NaCl, 60mM MgCl₂, 10mM DTT), MgCl₂ (50mM), Oligo(dT)₁₅ primers (0.5µg/µl), RNAsin (40,000U.ml), Taq DNA

polymerase (5U/ μ l), 10x polymerase buffer (500mM KCl, 100mM Tris-HCl, pH 9.0, 1% Triton X-100).

Strattech Scientific Ltd. (Luton, UK): 'GeneClean' DNA loading gel BBXG (bromophenol blue, xylene cyanole, glycerol).

iii) Primer Sequences

GM-CSF

GM-CSF primer sequences were designed from the human GM-CSF gene sequence (Kaushansky *et al* 1986) and were synthesised by the Imperial Cancer Research Fund, Clare Hall Laboratories, Potters Bar, UK: The sequences were as follows:

5'GGCTGGCCATCATGGTCAAG3' (MW 6580)

5'GAACCTGAGTAGAGACACTG3' (MW 6597)

These two primers defined a 170bp fragment which spans two introns (a 955bp genomic fragment). The concentration of primer in the stock solutions was measured and a working solution (50 μ g/ml) was prepared.

HPRT

HPRT primer sequences were obtained from Ewan Grant (Dept. Surgery, Western General Hospital, Edinburgh) and were designed from Gen Bank. The sequences were as follows (defining a 240bp fragment):

5'CTTGCTCGAGATGTGATGAAG3'

5'GTCTGCATTGTTTTGCCAGTG3'

Stock solutions of primers were stored at -20°C and the working solutions at 4°C.



2.2 Tissue Culture Techniques

a) Routine Cell Line Culture

All cell lines were routinely cultured in sterile 75ml culture flasks in a tissue culture incubator (37°C, humidified atmosphere and 5% CO₂ in air). The normal growth media varied according to the cell line, these are listed in table 3.

Table 3: Growth media used for routine cell line cultures.

Cell line	Normal growth medium
DU145, MG63, RT112, Hs578T, HF19, HEP2, ACHN, CHANG	1640 RPMI + 10% FCS (v/v)
LNCaP [†]	1640 RPMI + 10% FCS (v/v) + 10nM DHT* (Horoszewicz <i>et al</i> 1983)
PC-3	HAM's F12 + 7% FCS (v/v)

*DHT was prepared 10µM in ethanol and diluted 1:1000 (v/v) into medium.

[†]LNCaP cells showed no growth without 10nM DHT.

All the normal growth media were supplemented with 100U/ml penicillin, 100µg/ml streptomycin and 300mg/l glutamine. Cell cultures were twice monthly tested for mycoplasma using a kit from Boehringer Mannheim Biochemica (Germany).

b) Passaging Cell Cultures

Cell passage was required for plating cells (e.g., into 24 or 96 well plates), subculturing cells into two or more flasks (to increase cell number), or to freeze cells, for storage.

Medium was removed from cells growing in 75ml flasks (or petri dishes) and the cells were washed once with Dulbecco's A phosphate buffer. The

cells were then incubated for 5 minutes, at 37°C with 10% trypsin (diluted v/v with Dulbecco's A phosphate buffer). After this time (or when the cells had rounded up) the cells were harvested by washing them from the culture flask (or petri dish) with normal growth medium (10ml). The cell suspension was placed in a sterile universal and centrifuged at 360 xg for 5 minutes after which the supernatant was discarded and the pellet resuspended in the required medium. A single cell suspension was produced by titrating with a 10ml pipette. Cell numbers were counted and the cells seeded as required.

c) Primary Cell Culture

i) Human Prostate Epithelia and Fibroblast Culture

Media and Buffers

The following media were prepared:

Transport Media:

1640 RPMI
5% FCS (v/v)
100U/ml penicillin
100µg/ml streptomycin
300mg/l glutamine

Fibroblast Growth Medium:

1640 RPMI
10% FCS (v/v)
100U/ml penicillin
100µg/ml streptomycin
300mg/l glutamine

500ml Epithelial Growth Medium:

11.04g WAJC 404	5ml FCS
6.7g Hepes	1.2g NaHCO ₃
0.5mg insulin (from a working solution of 0.25mg/ml in distilled water)	
20µg cholera toxin (from a working solution of 10µg/ml in distilled water)	
1µM dexamethasone (from a working solution of 10mM in ethanol)	
10µg EGF (from a working solution of 1µg/ml in distilled water)	

Method

The following method was based on an earlier procedure by Chaproniere *et al* (1986) and was routinely used for the culture of fibroblasts and epithelia from human prostate.

'Chips' of human prostate were obtained from surgery after transurethral resections. These were collected in transport medium and either used immediately or stored overnight at 4°C (storage in this way had no apparent effect on culture). Parallel chips were sent to the Department of Pathology, Western General Hospital, Edinburgh, for histological examination.

Chips (1g) were aseptically dissected into small pieces and incubated with collagenase solution (1500IU collagenase dissolved in 7.5ml transport media), overnight at 37°C. The digested solution was titrated by repeated pipetting with a 10ml pipette and then centrifuged in a sterile universal, at 360 xg for 10 minutes, in a bench top centrifuge (Denley BS400, Billingshurst, UK). The supernatant was discarded, and the pellet was washed (to remove collagenase) in transport medium (20ml) and centrifuged as before. The supernatant was again discarded and the pellet was resuspended in transport medium (10ml) and centrifuged at 60 xg for 20 seconds. The resultant pellet contained mainly epithelial acini and the supernatant contained fibroblast aggregates.

The 'fibroblastic' supernatant was carefully collected and spun at 360 xg for 10 minutes. The resultant pellet was reconstituted with fibroblast growth medium (15ml) in a 75ml culture flask.

The 'epithelial' pellet was reconstituted into epithelial growth medium (15ml) and cultured in a 75ml culture flask.

Fibroblast cultures became confluent after approximately 2 weeks, and medium was replenished when required. Epithelial cultures usually adhered to the plastic after 5 days, therefore media could not be replenished until after this time. After 1 week in culture epithelial outgrowths were visible from acini.

ii) Human Osteoblast-like Cell Culture

The following normal growth medium was prepared:

Osteoblast Normal Growth Medium:	Dmem/F12 nutrient mix
	10% heat inactivated FCS (v/v)
	100U/ml penicillin
	100µg/ml streptomycin
	300mg/l glutamine

Osteoblast-like cells were cultured as described by Beresford *et al* (1984). Proximal femur or femoral heads were obtained from orthopaedic surgery, conveyed in transport medium (as for prostate primary culture) and used immediately for culture. Surgery was usually a hip replacement. Using sterile bone cutters, 0.5cm² chips of bone were excised. These were placed in a sterile universal and washed thoroughly using transport medium. Six - eight explants were placed in a sterile petri dish (10cm diameter), containing normal growth medium (20ml) and maintained at 37°C, in a humidified atmosphere of 5% CO₂ in air.

Growth media was renewed every week by the removal of 10ml of spent media and the addition of 10ml fresh media.

After 2 weeks, outgrowths of cells could be seen from the bone chips, by weeks 4-6 these usually became confluent. Once confluent, the cells were passaged into 75ml culture flasks and the remaining explants could be recultured for further use.

In this study individual osteoblast cultures were denoted by a capital letter (e.g., 'OBCM (A)').

iii) Human Skin Fibroblasts

A culture of human skin fibroblasts was kindly provided by Dr. Priestly (Dermatology, Royal Infirmary, Edinburgh). These were grown by incubating 1cm² sections of skin (from the buttock of an 85 year old female) in petri dishes containing 20ml normal growth media (Dmem/F12 nutrient mix, 10% heat inactivated FCS, 100U/ml penicillin, 100µg/ml streptomycin, 300mg/l glutamine) and maintained at 37°C, in a humidified atmosphere of 5% CO₂ in air. After approximately 2 weeks confluent cultures of fibroblasts were obtained.

d) Osteoblast Characterisation

The method of osteoblast cell culture used in this thesis, has already been widely characterised by Beresford *et al* (1983, 1984, 1986). For the purpose of this thesis osteoblast-like cultures were characterised, by the stimulation of alkaline phosphatase production with 10nM 1,25 (OH)₂ vitamin D (Murray *et al* 1987). Skin fibroblasts were used as a negative control since they are not sensitive to 1,25 (OH)₂ vitamin D in the above manner, whilst they have similar origins as the osteoblast i.e., both are connective tissue fibroblasts.

i) Histochemical staining of alkaline phosphatase

Method

Cells were passaged into 24 well plates (5000 cells/well) containing circular 1cm microscope slides. Once the cells became 50% confluent they were fixed with acetone (1ml) for 1 minute and then stained for alkaline phosphatase using a 'Sigma diagnostic kit'. The kit was based on the

incubation of cells with naphthol AS phosphate (pH 8.6, 0.25% w/v) as a substrate. Phosphatase activity produced naphthol AS. Subsequent incubation of the cells with fast blue RR, diazonium salt produced a coupling reaction which resulted in the formation of an insoluble, visible pigment at sites of enzyme activity.

Nuclear staining was achieved by incubation with Mayer's Haematoxylin (1ml) for 1 minute. Stained cells were then photographed directly in the 24 well plates using an Olympus IMT-2 inverted microscope.

The following cell cultures were stained for alkaline phosphatase; osteoblast-like cells grown in normal growth medium (plate 1a), osteoblast-like cells from the same culture, previously incubated for 48 hours in normal growth medium supplemented with 10nM 1,25 (OH)₂ vitamin D (plate 1b), skin fibroblasts grown in normal growth medium (plate 1c) and skin fibroblasts previously incubated for 48 hours in normal growth medium supplemented with 10nM 1,25 (OH)₂ vitamin D (plate 1d).

Plate 1a shows very little cytoplasmic staining, only the blue nuclear stain of haematoxylin. Plate 1b indicates cells with strong purple staining throughout the cytoplasm, indicative of alkaline phosphatase. Both plates 1c and 1d show very little cytoplasmic staining, only nuclear staining by haematoxylin.

These results indicate the osteoblast-like cells were stimulated by 10nM 1,25 (OH)₂ vitamin D to produce alkaline phosphatase. No such stimulation occurred with the skin fibroblasts, indicating that the osteoblast-like cells have osteoblast characteristics.

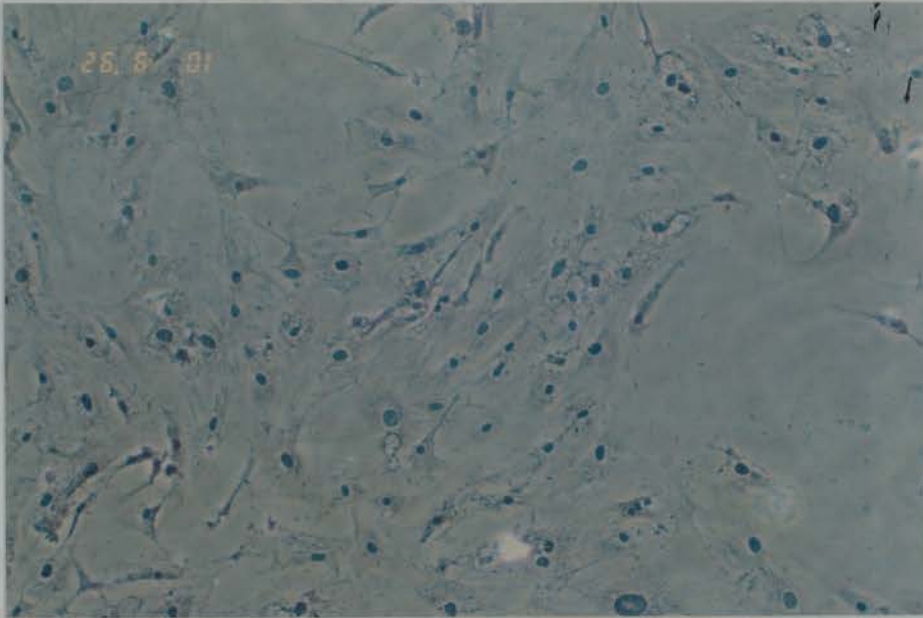


Plate 1a: Human osteoblast-like cells stained for alkaline phosphatase. x10 Magnification

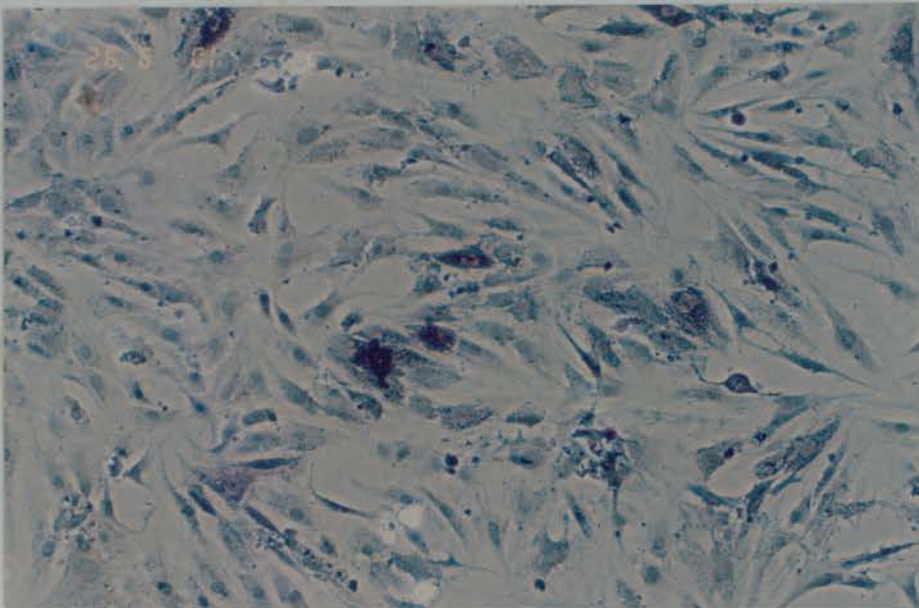


Plate 1b: Human osteoblast-like cells incubated in the presence of 10nM 1,25 (OH)₂ vitamin D and then stained for alkaline phosphatase. x10 Magnification

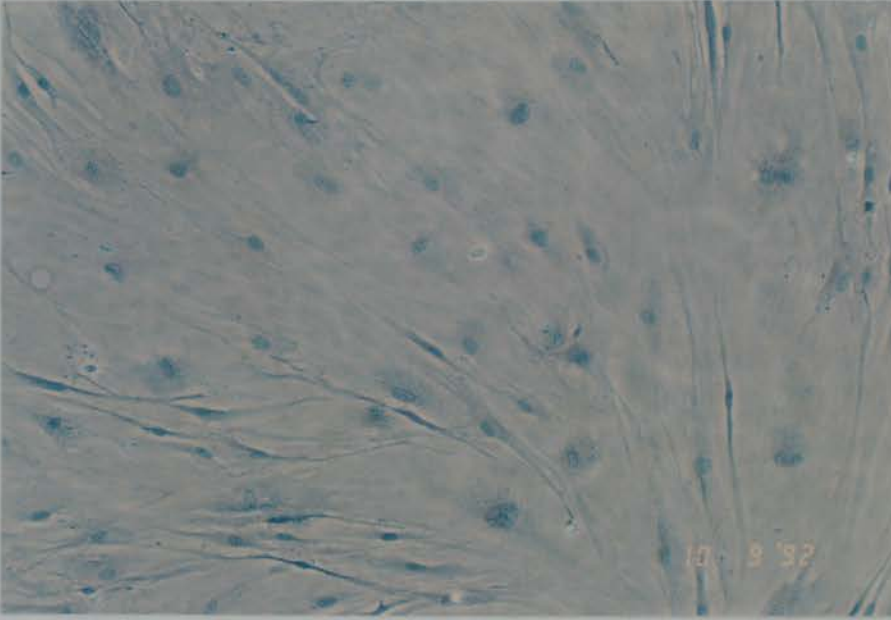


Plate 1c: Human skin fibroblasts stained for alkaline phosphatase. x10 Magnification

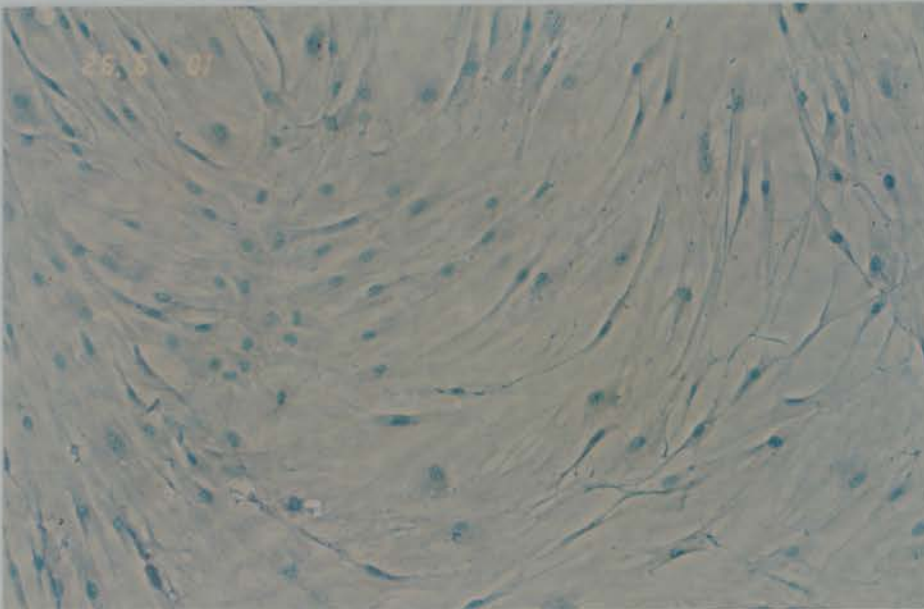


Plate 1d: Human skin fibroblasts incubated in the presence of 10nM 1,25 (OH)₂ vitamin D and then stained for alkaline phosphatase. x10 Magnification

ii) Colourimetric Assay for Alkaline Phosphatase

The production of alkaline phosphatase from osteoblasts, in response to 1,25 (OH)₂ vitamin D, was quantified using an alkaline phosphatase colourimetric assay.

Method

Osteoblast-like cells were plated into 24 well plates (10⁵ cells/well) and left overnight to adhere. The culture medium was replaced with normal growth medium with or without 10nM 1,25 (OH)₂ vitamin D. Cells were incubated in one of these media for a further 48 hours after which the media was aspirated and the cells frozen at -20°C for 15 minutes. The cells were then thawed at room temperature and assay buffer (200µl of 0.1M Glycine, 1mM MgCl₂, 0.1% Triton X-100 [v/v]) was added. This suspension was mixed by aspiration and 100µl was used to measure protein concentration, by reading the absorbance at 280nm. The remaining 100µl was used to assay alkaline phosphatase, by mixing with 0.01M p-nitrophenol phosphate (100µl, prepared in assay buffer) and incubating at 37°C for 60 minutes. The assay was stopped by the addition of 4M NaOH (20µl) and the absorbance of the solution was read at 405nm on a spectrophotometer (Pye Unicam 550, Cambridge, UK). The absorbance value was converted to alkaline phosphatase activity by comparison to a standard curve which had been prepared by measuring the absorbance of p-nitrophenol standards diluted in assay buffer at concentrations of 0.1-1µmol/ml.

The results from three different osteoblast-like cultures (V, RI and QI) are summarised in table 4 and compared to the quantities produced by human osteogenic sarcoma cell lines SAOS-2 and TE-85 (Murray *et al* 1987). They indicate that the production of alkaline phosphatase can be significantly stimulated by 10nM 1,25 (OH)₂ vitamin D. The level of this

activity varies with osteoblast-like cell culture, though V and QI show activity comparable to that of SAOS-2 cells. This confirms the histochemical results that the osteoblast-like cell cultures possess characteristics specific to osteoblasts.

Table 4: Colourimetric assay of vitamin D stimulated alkaline phosphatase activity from osteoblast-like cell cultures and cell lines.

Values are expressed as means (n=3) \pm sd. p values are shown if alkaline phosphatase activity is statistically different in cells treated with 1,25 (OH)₂ vitamin D in comparison to untreated cells.

Cell	Treatment	Alkaline phosphatase activity (nmol pNP/min/mg protein)
V Osteoblast-like	basal	1 509 \pm 202
	1,25 (OH) ₂ vitamin D	2 252 \pm 235 (p<0.0005)
RI Osteoblast-like	basal	7 290 \pm 290
	1,25 (OH) ₂ vitamin D	25 200 \pm 734 (p<0.0005)
QI Osteoblast-like	basal	1 268 \pm 101
	1,25 (OH) ₂ vitamin D	2 310 \pm 335 (p<0.005)
SAOS-2	basal	2 607 \pm 139
	1,25 (OH) ₂ vitamin D	2 712 \pm 30 (p<0.05)
TE-85	basal	16.8 \pm 1.4
	1,25 (OH) ₂ vitamin D	34.7 \pm 0.8 (p<0.0005)

e) Collection of Conditioned Media

i) Serum free medium (SFM)

SFM was used for collecting conditioned medium and, later, for reconstituting haematopoietic growth factors. This medium contained:

Dmem/F12 nutrient mix
100U/ml penicillin
100µg/ml streptomycin
300mg/l glutamine
10mg/ml insulin
10mg/ml transferrin
25nM sodium selenite.

ii) Collection of conditioned medium from cell lines, osteoblast-like cells and skin fibroblasts

Cells were grown to confluence in 75ml culture flasks. Growth media was then removed and the cells rinsed once with Dulbecco's A phosphate buffer. SFM (10ml) was exposed to the cells for 24 hours. This conditioned media was collected, filtered through a 0.2µm sterile filter and stored at -20°C (for no longer than 6 months) until used.

iii) Collection of Bone Marrow Conditioned Medium

The collection of bone marrow conditioned medium was based on the method used by Charkal-Roy *et al* (1989).

Fragments of proximal femora were obtained from orthopaedic surgery (as for the osteoblast culture). Using sterile bone clippers the red marrow was carefully removed and any bone fragments were gently washed with Dulbecco's A phosphate buffer and the washes added to the marrow. This mixture was centrifuged at 360 xg for 5 minutes. The pellet was resuspended

in serum free medium (10ml), placed in a 75ml culture flask and incubated for 24 hours at 37°C, in a humidified atmosphere of 5% CO₂ in air.

The resulting conditioned medium was clarified by centrifugation at 360 xg for 5 minutes and the supernatant filtered through a 0.2µm filter. The conditioned medium was then stored at -20°C (for no longer than 6 months) until used.

f) Reconstitution of Haematopoietic Growth Factors and Antibodies.

i) Haematopoietic Growth Factors (HGF)

All recombinant HGFs were provided in lyophilised form and were reconstituted using SFM plus 0.1% BSA (1ml). From these solutions 50µl aliquots were produced and stored at -70°C. The concentrations of HGFs in these aliquots were as follows:

rIL-3 of 1µg/ml (1000U/ml)

rG-CSF of 100ng/ml (10,000U/ml)

rGM-CSF of 1µg/ml (10,000IU/ml)

rEPO of 80µg/ml (10IU/ml)

ii) GM-CSF monoclonal antibody (Genzyme).

This antibody was purchased as 0.5mg in 0.5ml (1mg/ml), it was split into two aliquots of 0.25ml.

One aliquot was diluted by the addition of Dulbecco's A phosphate buffer plus 1% BSA to a concentration of 100µg/ml (1:10 dilution). These were aliquoted into volumes of 100µl and thereafter used for immunochemical staining.

The other 0.25ml aliquot was diluted by the addition of SFM plus 0.1% BSA to a concentration of 100µg/ml (1:10 dilution), aliquoted into volumes of 100µl and subsequently used for cell growth assays.

All the aliquots were stored at -70°C.

g) Growth Assay of Cell lines Following Exposure to Either Growth Factors or Conditioned Media

To assay the effect of growth factors or conditioned media on cell line growth, cells were exposed to the factor in serum free medium. The use of serum free medium avoided producing any unknown additive or conflicting effects serum may have on the growth factor action. DU145 and PC-3 cells both grew in serum free conditions however the LNCaP cell line did not. LNCaP cells were unable to grow in serum free media even in the presence of 10nM DHT. The LNCaP cell line showed very poor growth unless maintained in its normal growth medium. The assay procedures used were therefore different.

i) Plating and Preparation

DU145 and PC-3:

Cells were passaged into 96 well plates in normal growth medium (150µl), using a multipipette dispenser, at a density of 1000 or 2000 cells/well. Any wells that were unused were filled with growth medium to reduce evaporation. The cells were left overnight to plate, after which the medium was changed to serum free medium (150µl). The cells were grown for a further 48 hours to adjust to the serum free environment. After this time the test solution was added (150µl). The following growth factors were tested:

- recombinant haematopoietic growth factors (rIL-3, rG-CSF, rEPO and rGM-CSF) reconstituted and diluted in serum free medium containing 0.1% BSA.
- conditioned media, diluted in serum free medium.
- control cells were grown in either SFM or SFM plus 0.1% BSA, according to the test solution.

The cells were grown for a period of days in the test solutions, as detailed below, and cell numbers were subsequently measured by tritiated thymidine uptake.

LNCaP

Cells were passaged into 24 well plates, in normal growth medium (1ml), at a density of 5000 cells/well and allowed to plate for 48 hours. Plating required an extended period of time, in comparison to DU145 and PC-3 cells, since the LNCaP cell line adhered very loosely to plastic. After this time, test solutions (haematopoietic growth factors or conditioned media) were added, all of which were diluted in normal LNCaP growth medium. The cells were grown for a period of days as detailed below, with no media change. Cell growth was measured using cell counts. Thymidine uptake could not be used because large numbers were lost during the assay (since as stated above the cells adhered loosely to the plastic).

ii) Growth Assays

Dose Response assay

Cells were plated and grown, as above, then test solutions were added at varying concentrations by diluting in the appropriate medium (i.e., conditioned medium in SFM, haematopoietic growth factors in SFM plus 0.1% BSA and LNCaP test solutions in LNCaP normal growth medium).

After 3 days exposure to the test solutions DU145 and PC-3 cell growth was measured by tritiated thymidine uptake. LNCaP cell growth was measured by cell counts, after 5 days exposure to test solutions. At these time points the cells were judged to be in the logarithmic phase of growth, which for the prostate cell lines is between 30 and 80% confluence.

Time Course Studies (DU145 & PC-3)

The time dependency of stimulatory growth factor or conditioned medium was investigated as follows: cells were plated, as above, the test solution (150µl) was added (this was taken as day 0) and cell growth was assessed by tritiated thymidine uptake on day 0 and every subsequent 24 hours for 4 days (after this time cells were no longer in the logarithmic phase of growth). The test solutions were renewed after 3 days exposure to the cells.

Antibody Inhibition of GM-CSF Growth Stimulation

To test if the growth stimulation of DU145 cells by GM-CSF was specific to GM-CSF, the growth factor was pre-incubated with a monoclonal antibody raised specifically to human GM-CSF.

A solution of rGM-CSF was prepared at 200IU/ml in SFM. Several concentrations of antibody were also prepared - 4, 40, 400, 4000 and 40 000ng/ml (diluted with SFM plus 0.1% BSA). The rGM-CSF solution was mixed 1:1 (v/v) with each of the antibody solutions. This produced five solutions each containing 100IU/ml of rGM-CSF (a concentration known to stimulate prostate cancer cell lines) and an antibody concentration of 2, 20, 200, 2000 or 20 000ng/ml. These solutions were incubated at 37°C for one hour before addition to DU145 cells, plated as described in 2.2-g.i). Cells were grown for 3 days in these solutions, after which cell growth was measured by tritiated thymidine uptake.

The experiment was repeated exactly using mouse anti-human α fetoprotein (SAPU) as the monoclonal antibody.

h) Measurement of Cell Growth - Thymidine Uptake

Initially three assay methods were available to measure cell growth:

i) Cell counts with a haemocytometer

Described in section 2.6a

ii) ^3H -TdR Incorporation

Incorporation of tritiated thymidine into cellular material was assessed by washing cells (in their log phase of growth) with Dulbecco's A phosphate buffer. The cells were then incubated for 4 hours in methyl- ^3H thymidine diluted to 10 $\mu\text{Ci/ml}$ in serum free medium. After this time they were repeatedly washed in Dulbecco's A phosphate buffer and then incubated for 5 minutes with 10% trypsin (diluted v/v in Dulbecco's A phosphate buffer), followed by a 30 minute incubation with ice cold 10% TCA (w/v).

The cells were harvested with a 'Skatron Combi' cell harvester (Newmarket, UK) onto filter papers. These papers were dried, then punched into scintillation vials and dissolved in 6ml of 'Ultima Gold' scintillant. Disintegrations per minute were measured and calculated by a 'Canberra Packard' Tri-Carb liquid scintillation analyser.

iii) ^3H -TdR uptake

The measurement of tritiated thymidine uptake into cells was carried out on cells in their log phase of growth. The cells were washed once with Dulbecco's A phosphate buffer and then incubated for 4 hours in methyl- ^3H thymidine diluted to 10 $\mu\text{Ci/ml}$ in serum free medium. Following this incubation the cells were thoroughly washed in Dulbecco's A phosphate buffer and then incubated for 5 minutes in 10% trypsin (diluted v/v in

Dulbecco's A phosphate buffer). This produced a cell suspension which was harvested with a 'Skatron Combi' cell harvester (Newmarket, UK) onto filter papers. The filter papers were dried and then punched into scintillation vials and dissolved in 6ml of 'Ultima Gold' scintillant.

This scintillant completely removed all radioactivity from the filter after 5 minutes; this was confirmed by placing a filter paper with radioactive counts in a vial of scintillant, for 5 minutes, removing the filter and placing it in a second vial of scintillant. Counts were measured in the first vial and only background was detected in the second.

Counts per minute were then measured by a 'Canberra Packard' Tri-Carb liquid scintillation analyser. The analyser counted each sample for three minutes, corrected the counts for efficiency (quench) and calculated disintegrations per minute. The efficiency of the tritium channel remained at approximately 66% throughout the experiments.

iv)Choice of Assay

Each of the above methods was investigated for its suitability to assay cell growth in the experimental design.

Cell counts were not routinely used since a Coulter counter was not available and measurements had to be made with a haemocytometer. Counting in this way proved slow and inaccurate, due to human error.

Thymidine incorporation was found to be inaccurate at low serum concentrations. It was noticed that counts from thymidine incorporation were very low in serum free experiments even though there were cells in the wells. To investigate this, DU145 cell growth in serum free or 10% FCS medium was assayed using either thymidine uptake or incorporation. Any counts left behind (in the culture well) after harvesting were extracted with NaOH (0.5M

NaOH was added to the well, mixed with a pipette and removed into a scintillation vial and counted as before). Results are shown in table 5.

Table 5: Comparison of thymidine incorporation vs. thymidine uptake assays of DU145 cell growth in 10% FCS and serum free medium.

1000 DU145 cells were plated in normal growth medium and grown for 3 days in either 10% FCS or serum free medium. After this time they were incubated for 4 hours in tritiated thymidine (10 μ Ci/ml) and growth was measured either by thymidine uptake or by thymidine incorporation. Cells were then harvested and ^3H -TdR counted on a liquid scintillation analyser. For both assays the ^3H -TdR left behind in the wells was extracted using 0.5M NaOH, which was pipetted into a scintillation vial and counted as before. Values are expressed as means (n=6) \pm sd.

Cells grown in:	Thymidine assay	^3H counts (dpm) Thymidine	^3H counts (dpm) NaOH Extraction
Normal Medium (10% FCS)	Incorporation	2890 \pm 120	490
Normal Medium (10% FCS)	Uptake	2700 \pm 77	600
Serum free medium	Incorporation	435 \pm 48	1700
Serum free medium	Uptake	1741 \pm 130	450

When cells were grown in complete medium the dpm measured by both thymidine assays was similar (2890 and 2700) and very few dpm were left behind in the wells (490 and 600). However, when cells were grown in serum free medium the dpm measured by thymidine incorporation decreased dramatically to 435 dpm and the counts left behind in the well increased to 1700 dpm. Increasing trypsin concentration or trypsin incubation time did not increase the size of thymidine incorporation or decrease the counts found by NaOH extraction. Thymidine uptake still showed a high dpm count (1741) and low counts for the cells remaining in the well (450) when cells were grown in serum free medium. Similar experiments conducted in the absence

of cells did not indicate any counts above background levels for any of the three thymidine assays. These results indicate that TCA seemed to interfere with the action of trypsin or cell attachment in serum free environments, causing large numbers of cells to remain behind after harvesting. Therefore thymidine uptake was used to measure cell growth in a serum free environment.

Validation of Thymidine Uptake

To validate whether thymidine uptake could represent cell counts, prostate cell lines were plated and grown in serum free medium (as in section 2.2-g.i). Growth was subsequently assayed every 24 hours for a period of 4 days by either thymidine uptake or cell counts.

Figure 1 illustrates the growth of DU145 (a) and PC-3 (b) cell lines assayed by both thymidine uptake and cell counts. The graphs indicate that cell line growth over a period of 4 days followed a similar pattern, whether assayed by cell counts or thymidine uptake, i.e., as thymidine uptake increased so did cell counts. This, demonstrated thymidine uptake is a valid method for measuring cell growth when used in this experimental design.

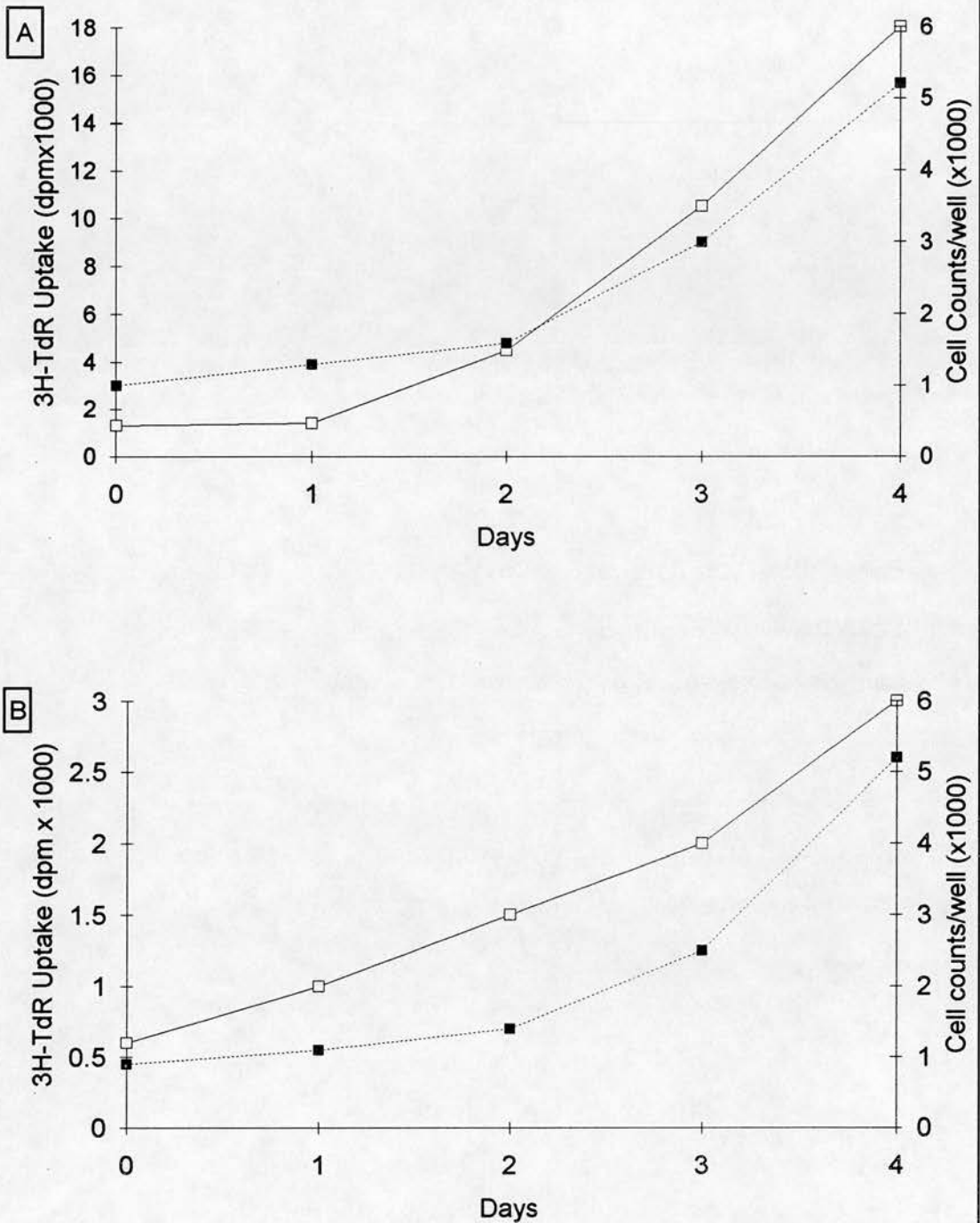


Figure 1a & 1b: Growth of Prostate Cancer Cell Lines In Serum Free Medium - Comparison of Growth Measurement by Cell Counts and Thymidine Uptake. DU145 [A] or PC-3 [B] cells were plated (1000/well) in normal growth medium. After 24 hours the medium was changed to SFM. The cells were incubated in SFM for 48 hours to adjust to the new medium. Growth was subsequently measured every 24 hours by both thymidine uptake (—) and cell counts (- -). Values are expressed as means (n=6). Standard deviations were <10% of the mean.

i) Characterisation Of Osteoblast-like Cell Conditioned Medium

i) Acid treatment

Four aliquots of OBCM (1ml) were each incubated with 1ml of citric-acid phosphate buffer (0.1M citric acid, 0.2M Na₂HPO₄) at one of four pH (2.6, 4.6, 6.6 and 7.6), for 2 hours at room temperature. At the end of the incubation serum free medium containing 1mg/ml BSA (9ml) was added to each solution to bring the pH back to 7.6. The mitogenic activity of acid treated OBCM were tested by incubating DU145 cells (plated as described in 2.2-g.i). in these solutions for 3 days.

ii) Heat treatment

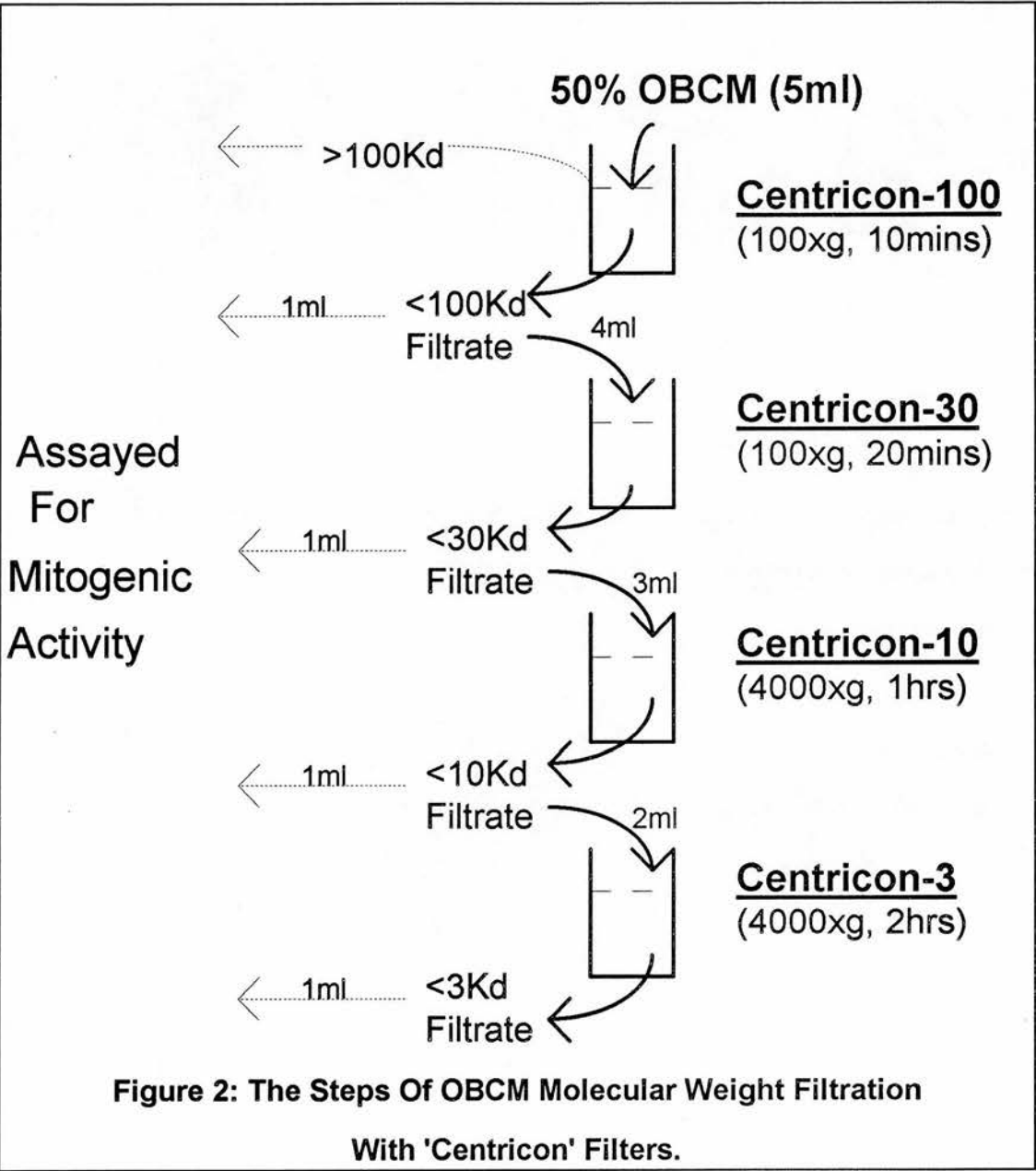
50% OBCM (5ml) was incubated, in a water bath, at 80°C for 1 hour, and then cooled to room temperature.

Each treated medium was tested for DU145 mitogenic activity, by exposing them to the cells (plated as described in 2.2-g.i) for 3 days.

iii) Molecular Weight Fractionation

OBCM was fractionated using 'centricon' filters, since gel filtration proved unsuccessful and high performance chromatography techniques were not available. The method is summarised in figure 2, and is described below.

50% OBCM (5ml) was loaded into a centricon-100 concentrator and centrifuged at 1000 xg for 10 minutes. The material retained on the filter (>100Kd) was recovered by the addition of 5mls of serum free medium to the sample reservoir (to run on a polyacrylamide gel this retentate was recovered in Dulbecco's A phosphate buffer). 1ml of the filtrate (<100Kd) was kept to assay its mitogenic activity and the rest was loaded into a centricon-30 concentrator and centrifuged at 1000 xg for 20 minutes.



Again, 1ml of the new filtrate (<30Kd) was kept to assay its mitogenic properties and the rest loaded into a centricon-10 concentrator and centrifuged at 4000 xg for 1 hour. This last step was repeated with a centricon-3 concentrator which was centrifuged at 4000 xg for 2 hours, and samples were retained for assay (<10Kd and <3Kd).

By the end of the filtration sequence five molecular weight fractions were produced, these were: >100Kd, <100Kd, <30Kd, <10Kd and <3Kd. Each fraction was tested for DU145 mitogenic activity, by exposing the cells (plated as described in 2.2-g.i) for 3 days to these solutions.

A sample of serum free medium (5ml) was similarly fractionated to provide five control fractions. A 1ml sample of each molecular weight fraction was later lyophilised and run on a 7.5% reducing polyacrylamide gel.

iv) OBCM incubation with monoclonal mouse anti-human GM-CSF

To determine whether or not GM-CSF was responsible for any of the stimulatory effects of OBCM, OBCM was pre-incubated with a monoclonal antibody raised to human GM-CSF (Genzyme).

OBCM was serially diluted with SFM to the following concentrations; 100%, 50%, 25%, 12.5% and 6.25%. Each dilution was mixed 1:1 (v/v) with anti-GM-CSF mAb solution (4µg/ml). This produced five solutions each containing 2µg/ml of anti-GM-CSF mAb and a dilution of OBCM as follows; 50%, 25%, 12.5%, 6.25% and 3.125%. A solution of SFM mixed 1:1 (v/v) with 4µg/ml of GM-CSF mAb was also prepared. All these solutions were incubated at 37°C for one hour before addition to DU145 cells, plated as described in 2.2-g.i). Cells were grown for 3 days in these solutions, after which cell growth was measured by tritiated thymidine uptake.

The above method was repeated by replacing the OBCM solution with a DU145 growth stimulatory fraction of OBCM (i.e., >100Kd or <100Kd).

2.3 Immunological Techniques

a) Immunohistochemistry

i) Solutions

The following solutions were prepared:

- **Tris Buffered Saline (TBS), pH 7.6:**

50mM	Tris
140mM	NaCl
0.1%	Triton X-100 (v/v)

- **TBS/BSA** (1% Bovine serum albumin [w/v] in tris buffered saline solution)

- **0.02M Imidazole Solution** prepared in distilled water.

- **1% & 3% H₂O₂** (v/v in distilled water)

- **1% formaldehyde** (v/v diluted in Dulbecco's A phosphate buffer)

- **5% acetic acid** (v/v in ethanol)

- **Primary Antibody Solution**

monoclonal mouse anti-human GM-CSF (IgG₁) was diluted in TBS/BSA, to the required dilution.

- **Secondary Antibody Solution**

sheep anti-mouse (Fab fragments conjugated to peroxidase) was diluted 1:40 (v/v) in TBS/BSA solution.

- **DAB Solution (diaminobenzidine hydrochloride):**

DAB (2mg) was dissolved in TBS (2ml), to this was added 0.02M imidazole (60μl) solution and the mixture was heated to 37°C. Finally 1% H₂O₂ (30μl) was mixed into the solution.

ii) Preparation and fixation of cell lines and primary cultures

Cells were passaged into 24 well plates in normal growth medium (1ml) at a density of 10 000 cells/well. When the cells were approximately 50% confluent they were fixed in 1% formaldehyde (250μl) for 20 minutes at room

temperature, followed by 5% acetic acid (250µl) for 10 minutes. The cells were then washed twice in TBS, each wash was left for 3-4 minutes.

iii) Preparation of Frozen Tissue Sections

Microscope slides (Chance Propper Ltd., Warley, UK) were first prepared by cleaning them with acid alcohol (1% HCl [v/v] in 70% Ethanol) and then incubating in a L-lysine solution at room temperature for 5 minutes. These were then either air dried overnight or at 60°C for 1 hour.

The slides were used to mount 5µm cryostat frozen (-70°C) tissue sections. These were fixed by incubating in acetone for 10 minutes and then air drying for 5 minutes and finally rinsing in TBS.

iv) Preparation of Paraffin-embedded tissue sections

Paraffin embedded tissue sections were obtained from the Pathology Dept., at the Western General Hospital, Edinburgh. This provided prostate tissue sections which had been examined by pathologists and their Gleason scores (Foster 1992) obtained. In this study, well differentiated cancers have a Gleason score ≤ 4 , moderately differentiated 6 and poorly differentiated >6 .

The sections were first dewaxed by heating to 60°C for 15 minutes, after which they were washed twice in xylene for 3 minutes, at room temperature. This was followed with repeated three minute washes in 100% ethanol, and then repeated washes in 95% ethanol, both at room temperature. The sections were finally rinsed in TBS.

v) Immunostaining

Fixed tissue sections or cell lines were all stained according to the method described below.

Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide for 30 minutes, at room temperature. Samples were then washed twice in TBS, each for 5 minutes. Non-specific sites were blocked by

incubation in a solution containing 20% sheep serum (v/v) and 20% human male serum (v/v) diluted in TBS/BSA solution, for 1 hour at room temperature. The blocking solution was drained off and the samples incubated in primary antibody. Titration of the primary antibody, indicated that positive staining occurred between dilutions of 1:100 and 1:800. Optimal staining was as follows:

- Cell lines: incubated in a 1:200 dilution of primary antibody (v/v) in TBS/BSA solution, overnight, at 4°C, inside a humidified container.
- Tissue sections: incubated in a 1:400 dilution of primary antibody (v/v) in TBS/BSA solution for 2 hours at room temperature, inside a humidified container.

TBS/BSA solution was used as a negative control. After incubation with the primary antibody, samples were washed thoroughly in TBS and then incubated with the secondary antibody, for 1 hour, at room temperature inside a humidified container. Samples were again washed repeatedly in TBS and then incubated in DAB solution for 5 minutes (or until colour developed), at room temperature.

Samples were rinsed in tap water, and then incubated in Mayer's haematoxylin for 1 minute, at room temperature, followed by 1 minute in saturated lithium carbonate.

Cover slides (Chance Proper Ltd.) were applied to tissue sections using DePeX, and these were photographed using a Leitz Ortholux 2 light transmitting microscope.

Cell lines were photographed directly in the 24 well plates using an Olympus IMT-2 inverted microscope.

vi) Specificity of Immunostaining

The specificity of immunostaining produced by the GM-CSF monoclonal antibody was investigated by pre-incubating the primary antibody with excess rGM-CSF, prior to staining the DU145 cell line. The primary antibody was used at a dilution of 1:800. Titration had shown this to be the lowest dilution of antibody that still produced a positive result, therefore this reduced the amount of GM-CSF required to adsorb all the antibody.

A 1:400 dilution of primary antibody was prepared (with TBS/BSA) and mixed 1:1 (v/v) with a solution of rGM-CSF (1250U/ml \equiv 125ng/ml). This produced a final solution containing a 1:800 dilution of primary antibody and rGM-CSF (625U/ml), this concentration of rGM-CSF was calculated to be 15 times the molar excess of the antibody. This mixture was incubated at 37°C for one hour and then used as the primary antibody solution for immunostaining.

In a similar experiment the antibody was also pre-incubated with excess IL-3 (125ng/ml) and excess EPO (125ng/ml), to act as controls. As a blank, antibody solution (1:800) was incubated 1:1 (v/v) with SFM plus 0.1% BSA. Negative staining was achieved by replacing the antibody solution with TBS/BSA and mixing 1:1 (v/v) with SFM plus 0.1% BSA.

b) Enzyme Linked Immunoassay (ELISA)

The concentration of GM-CSF in the conditioned media of various cells was measured using a Quantikine Kit for human GM-CSF. The principles of this kit were based on a 'quantitative immunometric, sandwich enzyme immunoassay'.

A microtiter plate was provided, previously coated with a mouse monoclonal antibody to GM-CSF. GM-CSF standards were pipetted into the wells along with undiluted samples of conditioned media (3 wells/sample or standard). Any GM-CSF present in the media, bound to the plate and unbound proteins were washed away. A second goat monoclonal antibody (conjugated to peroxidase) was added to the cells and adsorbed to the bound GM-CSF. After washing away unbound antibody a substrate solution was added which contained a chromogen (tetramethylbenzidine), and colour developed according to the amount of GM-CSF present. This was measured as an absorbance at 450nm. The minimum detectable dose of the assay was 1.5pg/ml GM-CSF.

The antibody was cross reacted with TGF α (0.5 μ g/ml), TGF β (10.5ng/ml), EPO (80ng/ml), IL-3 (10ng/ml) and G-CSF (100ng/ml), to check its specificity.

The ELISA kit was used to assay media conditioned by; DU145 cells, PC-3, LNCaP, benign prostate fibroblasts, MG63 and osteoblast-like cells.

The absorbance at 450nm was measured using a Bio-Rad 450 microplate reader (Watford, UK), which used a correction wavelength of 540nm. The plate reader calculated the standard curve and data.

2.4 Western Blotting

a) Solutions

The following solutions were prepared using electrophoresis grade chemicals:

Phosphate Buffer (pH 7.6)

Prepared with distilled water

8mM Na₂HPO₄

2mM Na(HPO₄)₂

i) Polyacrylamide gel electrophoresis

30ml Resolving Gel	12% (ml)	7.5% (ml)
H ₂ O	12.9	16.2
40% acrylamide	9.0	5.6
1.5M Tris (pH 8.8)	7.5	7.6
10% SDS (w/v)	0.3	0.3
10% ammonium persulfate (w/v)	0.3	0.3
TEMED	0.012	0.012

10ml, 5% Stacking Gel	(ml)	Tris-Glycine Electrophoresis Buffer	
H ₂ O	7.3	Prepared with distilled water	
40% acrylamide	1.25	25mM	Tris
1.5M Tris (pH 6.8)	1.25	200mM	Glycine
10% SDS	0.1	3.5mM	SDS
10% ammonium persulfate	0.1		
TEMED	0.01		

ii) Blotting

Transfer Buffer (pH9.2)

Prepared with distilled water
48 mM Tris
9mM Glycine
20% methanol (v/v)

iii) Immunostaining

Washing Solution (pH 7.5)

Prepared with distilled water
50mM Tris
150mM NaCl
2mM EDTA

Blocking Solution

5% skimmed milk (w/v) and 2% BSA (w/v) dissolved in washing solution

Primary Antibody Solution

Monoclonal mouse anti-human GM-CSF (Genzyme) or polyclonal sheep anti-human GM-CSF serum (National Institute For Biological Standards and Controls) were diluted 1:100 (v/v) with blocking solution.

Secondary Antibody Solution

(for the primary, polyclonal sheep anti-GM-CSF)

Donkey anti-sheep IgG peroxidase conjugate (Boehringer Mannheim Biochemica) was diluted 1:1000 with blocking solution.

iv) Silver Staining

All water used was the purest available

Silver solution

Two solutions were prepared:

- a) 0.8g AgNO₃ in 4mls distilled H₂O
- b) 1.4ml conc. NH₄OH plus 21ml 0.36% NaOH (w/v).

Solution (a) was mixed vigorously to (b) and the volume made up to 100ml with distilled water

Developer

0.12 mls 37% formaldehyde
1.25ml 1% citric acid (w/v)
148.63ml distilled water.

Stop Solution

50% distilled water, 45% methanol and 5% acetic acid (v/v/v)

b) Sample preparation

Conditioned media were collected from DU145, PC-3, LNCaP, MG63, benign prostatic fibroblasts and osteoblast-like cells (as in section 2.2.e). Salts and small molecular weight compounds were removed from the conditioned media by dialysis against 5 litres of 10mM, pH 7.6 phosphate buffer using Spectrapore 3 dialysis tubing (molecular weight cut off 3.5Kd). Buffer was changed twice, over 24 hours. The protein concentrations of the dialysed media were measured using the Bradford assay and a sample of

each was snap frozen in liquid nitrogen and lyophilised to dryness at a pressure of 0.1mbar.

The powdered media were reconstituted in Dulbecco's A phosphate buffer to a concentration of 0.1-1mg protein/ml. Samples were mixed 1:1 (v/v) with 'Blue juice' sample buffer and stored at -20°C or used directly for electrophoresis.

c) SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

PAGE was based on the method described by Laemmli (1970) using a Bio-Rad Protean II apparatus. All parts of the casting mould were cleaned with 70% ethanol before assemblage. The components of the resolving gel were mixed in the order listed (ammonium persulfate prepared fresh) into a 50ml Falcon tube. As soon as TEMED was added the contents of the tube were carefully mixed and poured. The gel was overlaid with water-saturated isobutanol to a 1cm depth and the gel was left to polymerise for 45 minutes. Once the gel had set, the overlay was removed using the edge of a clean filter paper and rinsed with distilled water.

Components of the stacking gel were carefully mixed, in the order listed, into a 50ml disposable falcon tube, and poured immediately onto the resolving gel. The sample comb was carefully inserted into the stacking gel solution and left to polymerise for a further 45 minutes. After polymerisation was complete the comb was removed and using a squirt bottle, the wells were washed with electrophoresis buffer to remove unpolymerised acrylamide. The gel was then placed in the electrophoresis apparatus and electrophoresis buffer was added to the top and bottom reservoirs.

Samples previously prepared in 'Blue juice' loading buffer were boiled for 5 minutes to denature the proteins, and cooled. Samples (10-30µl) were loaded into the wells with a Hamilton syringe (1-10µg of a polypeptide and

50-100µg of a protein mixture). Rainbow molecular weight markers (20µl) were similarly loaded (range: 2.35-46Kd or 14.3-200Kd) and rGM-CSF (25ng) was loaded as a positive control. The power source was attached and the electrophoresis was run at 20mA until the dye reached the resolving gel and then turned up to 40mA, until the dye was 1cm from the gel bottom. After electrophoresis was complete the gel was used for Western Blotting or silver staining.

d) Western Blotting

i) Blotting

The gel was placed in transfer buffer and soaked for 30 minutes. After equilibration, the gel was measured and one piece of nitrocellulose membrane, plus four pieces of 3MM Whatman paper were cut to the same dimensions. The membranes and papers were also equilibrated for a further 30 minutes. Blotting took place in a Bio-Rad Trans Blot semi dry electrophoretic transfer cell. Two pieces of Whatman were placed exactly on top of each other onto the anode and any air bubbles rolled out. The nitrocellulose was then placed on top of the Whatmann papers, then the gel and then the remaining 2 pieces of Whatman. The cathode was placed on top and blotting was carried out overnight at 5V.

ii) Immunodetection

The nitrocellulose membrane was removed from the blocking apparatus and briefly rinsed with washing solution. It was then incubated in blocking solution for 1 hour at room temperature, on a shaking platform (0.1ml solution/cm² membrane) in a heat sealable bag. After this, the membrane was washed thoroughly in washing medium and then incubated with one of the primary antibody solutions (0.1ml solution/cm² membrane), for 2 hours at

room temperature. The membrane was repeatedly washed in washing medium. Antibody detection was dependent on the primary antibody, as follows:

- mouse monoclonal anti-GM-CSF detection was performed with the ECL Western Blotting analysis system. Briefly, this system incubated the membrane with a 1:1000 dilution of secondary antibody (sheep anti-mouse and conjugated to peroxidase) for 1 hour. The membrane was washed thoroughly and a detection reagent added for 1 minute to the side of the membrane carrying the protein.
- polyclonal sheep anti-GM-CSF was detected by incubating the membrane with a donkey anti-sheep secondary antibody solution for 1 hour. The membrane was then washed thoroughly and the ECL detection reagent added for 1 minute to the side of the membrane carrying the protein.

The membrane was wrapped in saran wrap and exposed to Hyperfilm-ECL (placing the protein side of the membrane against the film) for 5 minutes. Lastly the film was developed with a Kodak RP X-OMAT processor.

e) Silver Staining

The gel was fixed overnight in 50% methanol (v/v with distilled water) followed by incubation in the silver solution for 15 minutes on an orbital shaker, at room temperature. The gel was then washed extensively in water. Developer was added until proteins bands were easily visible and then the reaction was stopped by incubation with the stop solution.

The gel was finally placed in a heat sealable bag with a small amount of stop solution and photographed immediately.

2.5 Molecular Biology Techniques

a) Solutions

i) RNA Extraction

To destroy RNase contamination, all solutions were incubated overnight with depc (20µl depc/100ml of solution) followed by autoclaving. Glassware was similarly treated with depc, by rinsing inside the glassware with a depc solution (20µl depc/100ml water) and then autoclaving. All the plastics used were sterile and free of RNases.

The following solutions were prepared:

GTC Denaturing Solution

0.318ml	Sodium citrate (0.75M, pH 7.0)
0.476ml	Sarcosyl (10% w/v)
5.54ml	Depc treated H ₂ O
4.73g	Guanidine isothiocyanate
0.072ml	Mercaptoethanol

2M Sodium Acetate (anhydrous), pH 4:

Prepared with 32% depc water and 68% glacial acetic acid (v/v)

ii) Polymerase chain reaction

Tris Borate Buffer (TBE)

90mM	Tris base
90mM	Boric acid
2mM	EDTA (pH 8.0)

b) RNA Extraction

RNA extraction was based on a method used by Chomczynski and Sacchi (1987). Where possible the experiments were carried out on ice to reduce any contaminating RNase activity.

i) Preparation of Cell lines

Cells were grown to confluency in 75ml tissue flasks (approximately 1.5×10^6 cells). These were then rinsed with sterile Dulbecco's A phosphate buffer, and drained as much as possible. Denaturing solution (2ml) was added to the flask and incubated with the cells for 30 minutes on an orbital shaker at 4°C. This solution was then drained into a sterile 10ml falcon tube.

ii) Preparation of Blood Cells

Total female blood (10ml) was incubated on ice with denaturing solution (10ml) for 1 hour and then placed in a 50ml sterile falcon tube.

iii) Extraction

Cells in denaturing solution were homogenised to sheer the DNA, after which the foam was allowed to settle on ice for 1 hour, the solution was then transferred in 500µl aliquots to sterile eppendorfs.

Sodium acetate (50µl of 2M, pH4) was added to each eppendorf and vortexed, then water saturated phenol (500µl) was added and vortexed. Finally, chloroform/isoamyl alcohol (100µl, 49:1[v/v]) was added and vortexed vigorously for 15 seconds. Finally, the eppendorfs were spun in a bench top microfuge (MSE, Microcentaur, MacKay & Lynn Distributors, Edinburgh, UK) for 20 minutes at 14 000 xg (4°C).

This produced two phases. Using a pasteur pipette, the top aqueous phase was transferred to fresh sterile eppendorfs, to which isopropanol (200µl) was added. This solution was vortexed and stored at -20°C for 1 hour to precipitate the RNA. After precipitation, the RNA was spun in a microfuge for 20 minutes at 14 000 xg (4°C). The supernatant was carefully removed and the pellet resuspended in denaturing solution (60µl). At this point aliquots were pooled and isopropanol was added 1:1 (v/v). This was

precipitated, overnight at -20°C after which it was spun for 10 minutes at 14 000 xg (4°C).

The supernatant was removed and the pellet washed in 75% ethanol. At this point the pellet could be stored at -70°C (in 75% ethanol) or resuspended in depc water. To re suspend in water, the RNA was spun for 10 minutes at 14 000 xg (4°C), and the ethanol removed with a pipette. The pellet was then air dried for 1 hour (or under vacuum for 5-10 minutes). This formed a translucent pellet which was resuspended in 11µl of depc water. The concentration of RNA in the solution was then calculated.

c) Reverse Transcription

Total RNA (1µg) was reverse transcribed in a reaction volume of 20µl, by the addition of:

1µl	RNA (1µg)
2µl	dNTP mix (10mM)
0.5µl	RNasin (40 000U/ml)
1µl	Oligo (dT) ₁₅ primers (500ng/ml)
0.8µl	AMV reverse transcriptase (25 000U/ml)
4µl	AMV RT Reaction buffer (5x)
4µl	MgCl ₂ (25mM)
6.7µl	Autoclaved water

The solution was mixed by gentle flicking and incubated at 42°C for 60 min.

d) Polymerase Chain Reaction (PCR)

i) Reaction

Amplification of specific RNA sequences was carried out in an microtest-tube in a reaction volume of 100µl, which contained:

20µl	reverse transcriptase mix
16µl	dNTP mix (1.25mM)
0.2µl	Taq DNA polymerase (5000U/ml)
2x10µl	primers (50µg/ml) - approximately 75pmol of each
8µl	assay buffer (10x)
31.8µl	autoclaved water

The reaction mix was vortexed and mineral oil (50µl) was layered on top, and pulsed in a microfuge. Using a Hybaid Thermal Reactor (Teddington, UK) reaction mixtures underwent denaturation at 94°C for 1 minute, annealing at 55°C for 1.5 minutes and extension at 72°C for 3.5 minutes, for a total of 40 cycles. The products were then size separated on a 2% agarose gel.

ii) cDNA Digestion with *Bgl* I

GM-CSF PCR products were characterised by the ability of *Bgl* I to cleave the 170 bp product into two smaller products of 123 and 47 bp. A restriction digest was carried out by mixing:

20µl	PCR product
5µl	Enzyme buffer (10x)
24µl	Autoclaved water
1µl	<i>Bgl</i> I

This mixture was vortexed and then incubated at 37°C for 1 hour.

iii) Analysis of Genomic DNA contamination

In order to demonstrate that GM-CSF PCR products were due to RNA and not genomic DNA the presence of genomic contamination was analysed by amplifying 1µg RNA without reverse transcription. Therefore the 20µl of RT mix in the PCR reaction was replaced with a solution containing 1µg

RNA and amplified as before. Any genomic contaminants would produce a 955 bp product, since the GM-CSF primers span two introns.

iv) Size Fractionation Of PCR Products

PCR products were size fractionated on a midi gel apparatus (Northumbria Biologicals Ltd., Cramlington, UK). This was assembled after cleaning with 75% ethanol and rinsing in TBE.

A 2% gel (w/v) was cast by melting agarose in TBE (70ml), using a microwave oven. While the solution was cooling 1µl of ethidium bromide was added. When the gel solution reached hand heat it was poured into the gel mould and left to set for 30 minutes. Once set, the comb was removed and the gel was placed in the tank filled with TBE.

PCR product (27µl) was mixed with DNA gel loading dye (3µl) and loaded into a sample well using a Gilson pipette along with a 100 base pair ladder (30µl).

The gel was then run at 50V for 4-5 hours after which it was illuminated by a TM-20 transilluminator (UVP Ltd., Cambridge) and photographed with polaroid instant film (type 667).

2.6 Routine Methods

a) Cell Counts

The number of viable cells was counted by mixing 0.5ml of single cell suspension to 0.5ml of trypan blue (0.5% NaCl + 0.5% Trypan blue, w/w/v in distilled water). A sample of this solution was then carefully loaded onto a haemocytometer and the viable cells were counted (non-viable cells stained blue). Cell number was calculated using the following equation:

$$(\text{No. cells counted}/\text{No. squares on haemocytometer counted}) \times 20^4$$

b) Protein Analysis

i) Bradford assay (Bradford 1976)

A stock solution of BSA (160µg/ml) was prepared and serially diluted to give 1ml volumes of 16, 12, 8, 6, 4, 2µg/ml standards, these were used to produce a standard curve. Distilled water or reaction medium was used as a blank and for diluting, as appropriate.

Each standard or sample (0.8ml) was added to Bradford reagent (0.2ml) and vortexed (usually carried out in triplicate). These solutions were left for a period of 5-60 minutes after which the absorbance at 595nm was read on a spectrophotometer (Pye Unicam 550, Cambridge, UK).

Samples were diluted and added to reagent so that their absorbance values fitted on the curve (and also to check that dilution of the sample led to dilution of protein concentration measured). Protein concentrations were read from the standard curve.

ii) Absorbance at 280nm

If a sample contained a chemical which interfered with the Bradford reagent (i.e., if the diluent did not produce a standard curve or produced an emulsion), protein concentration was measured at 280nm. Solutions for a standard curve were prepared as for the Bradford assay.

c) Quantification of RNA and DNA

Samples of RNA or DNA solutions (1 μ l) were added to depc treated water (1ml). The absorbance of this solution was then read in a spectrophotometer (Pye Unicam 550, Cambridge, UK) at 260nm or 280nm. Concentrations were calculated as indicated below.

i) RNA

Using the following equations the concentration and purity of RNA solutions were calculated:

At 260nm one OD unit is equivalent to 40 μ g/ml of RNA

Pure RNA samples have a 260/280 absorbance ratio of 1.8-2.0

(values below 1.6 indicate the sample is badly contaminated with protein).

ii) DNA

The concentration of DNA samples were calculated as follows:

At 260nm one OD unit is equivalent to 50 μ g/ml of double stranded DNA.

At 260nm one OD unit is equivalent to 40 μ g/ml of single stranded DNA.

iii) Oligonucleotide

The concentration of oligonucleotide samples were calculated as follows:

At 260nm one OD unit is equivalent to 20 μ g/ml of single stranded oligonucleotide.

d) Statistics

To test whether or not the means of two independent groups were significantly different, statistics were calculated using the students unpaired t-test to calculate p values. This test assumed that the samples analysed had normal distributions. If the samples were judged to have non-parametric distributions then statistics were calculated with the Mann-Whitney test. Significant p values were <0.05 .

CHAPTER 3

RESULTS

The results in this chapter are presented in the following sections:

- 3.1 The effects of osteoblast-like cell conditioned medium on prostate cell lines.
- 3.2 Characterisation of osteoblast-like cell conditioned medium.
- 3.3 The effects of haematopoietic growth factors on prostate cell lines.
- 3.4 Evidence that GM-CSF may or may not be responsible for the OBCM stimulation of prostate cell lines.
- 3.5 Secretion of GM-CSF by prostate cell lines.

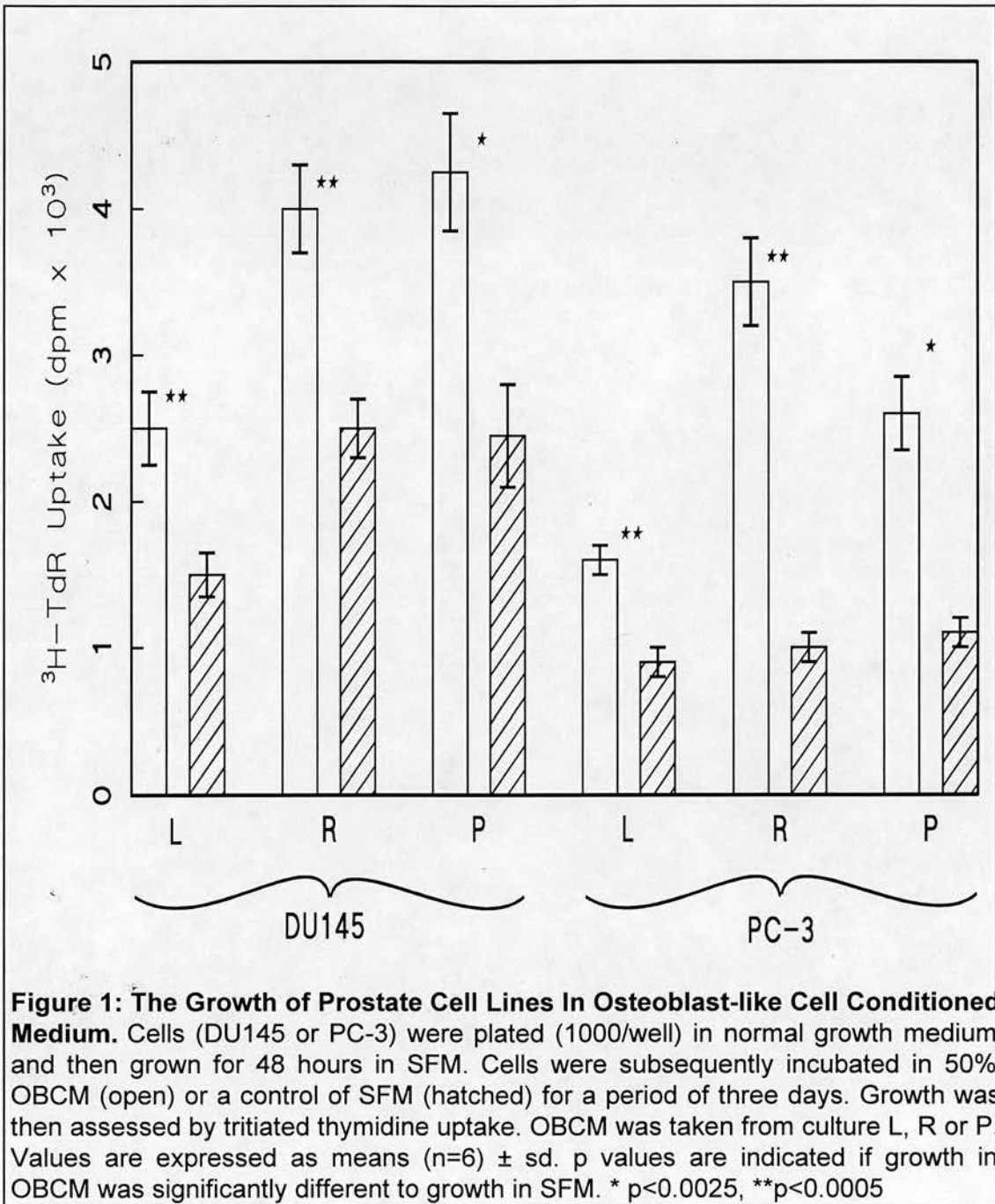
3.1 The Effects Of Osteoblast-like Cell Conditioned Medium On Prostate Cell Lines

a) Growth of prostate cancer cell lines in osteoblast-like cell conditioned medium and bone marrow conditioned medium

In order to understand how prostate cancer is stimulated by factors in the bone environment, initial experiments looked at the effects of media conditioned (CM) by primary cultures of human osteoblast-like cells (OB) and bone marrow (BM), on the growth of prostate carcinoma cell lines.

PC-3 and DU145 cells were incubated for 3 days in the presence of OBCM (figure 1) or BMCM (figure 2), both diluted to 50% with serum free medium. Following the incubation, growth was measured by tritiated thymidine uptake and compared to growth in serum free medium.

Figure 1 shows the effects of CM collected from three different osteoblast cultures (L, R, P). All stimulated the growth of both DU145 and PC-3 cells when compared to controls of serum free medium. Stimulations were in the order of 1.8 to 3 fold, and all were statistically significant, p values are indicated on the graph.



The results in table 1 summarise all the studies that have investigated the effect of 50% OBCM on DU145 and PC-3 cell growth. Stimulation was observed from all the five conditioned media tested. The table shows that media conditioned by one individual osteoblast culture could reproducibly and repeatedly stimulate both prostate cell lines. All OBCM stimulated DU145 growth roughly by the same degree, this stimulation had a mean (\pm sd) value of 2.3 (\pm 0.3) fold. PC-3 cells were also stimulated by approximately the same degree, the mean value of which was 3.2 (\pm 0.6) fold. These results demonstrate that PC-3 cells were usually stimulated to a greater extent than DU145 cells. Statistical analysis revealed that the mean value of PC-3 cell growth stimulation was significantly ($p < 0.0005$) greater than that of DU145 cells.

In contrast to OBCM, media conditioned by red bone marrow had no significant ($p > 0.05$) effects on the growth of DU145 or PC-3 cells (figure 2). This effect was reproduced with media conditioned by three different bone marrow cultures (V, P, T).

Previous work by Charkal-Roy *et al* (1989) showed that BMCM did stimulate prostate cell line growth in the presence of 1% FCS. Therefore, the experiment illustrated in figure 2 was repeated, but the BMCM was supplemented with either 1% FCS or 5% FCS, to establish whether or not FCS could produce synergistic growth effects with BMCM. However, as the results in figure 3 indicate when 'BMCM (V)' was supplemented with 1% or 5% FCS there were still no stimulatory effects on DU145 growth.

Table 1: The Growth of Prostate Cell Lines In Osteoblast-like Cell Conditioned Medium.

Cells were grown in 50% OBCM as indicated in the legend of figure 1.
Values are expressed as means (n=6). Standard deviations were <15% of the mean.

Cell Line	OB Culture	OBCM (dpm)	Control (dpm)	Stimulation (CM/Control)
DU145	R	2200	900	2.4
		3300	1500	2.2
		4000	2500	1.6
		3200	1400	2.3
	P	2050	1000	2.05
		1700	900	1.9
		2400	1000	2.4
		4400	2500	1.8
	L	2500	1500	1.7
	O	2400	900	2.7
	Q	3900	1400	2.8
PC-3	R	3500	990	3.5
		4500	1200	3.75
		3500	1100	3.2
		3200	990	2.2
	P	3500	900	3.8
		3500	990	3.5
		4300	1200	3.5
		2500	1200	2.1
	L	1600	900	1.8
	O	3900	1000	3.9
	Q	3200	1000	3.2

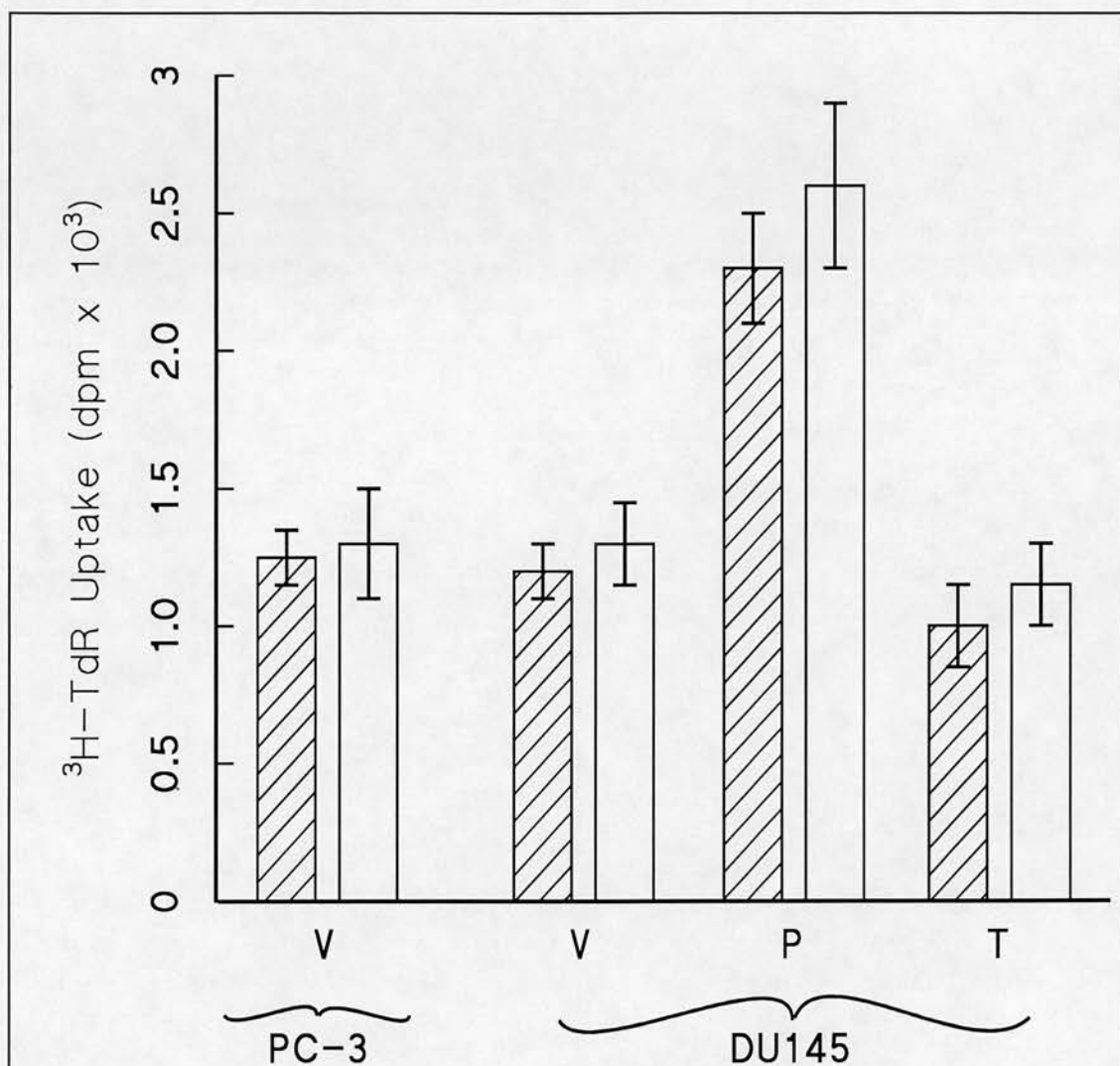
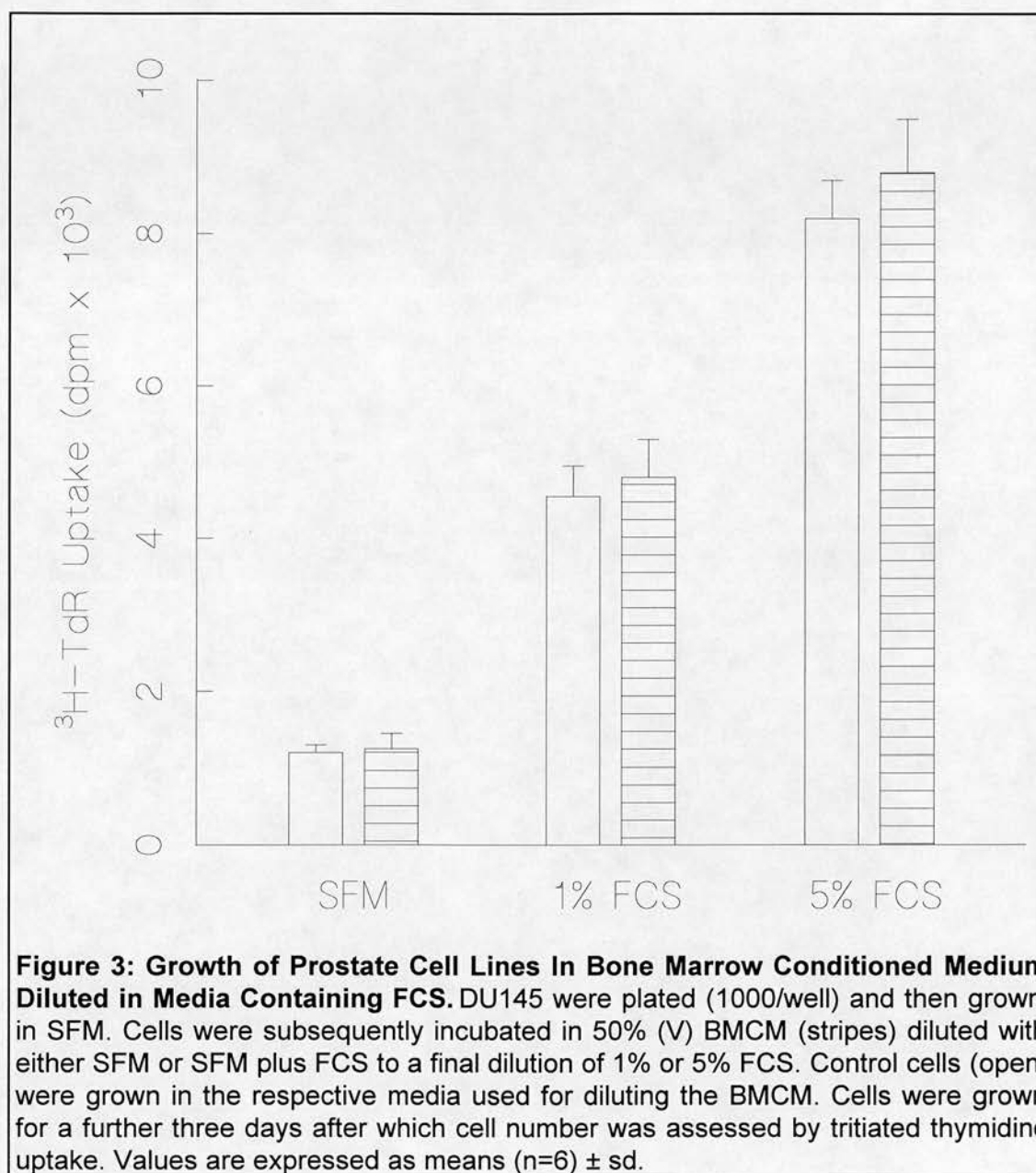


Figure 2: The Growth of Prostate Cell Lines In Bone Marrow Conditioned Medium. Cells (DU145 or PC-3) were plated (1000/well) and then grown in SFM. Cells were subsequently incubated in 50% BMCM (hatched) or a control of SFM (open) for a period of three days. BMCM was collected from culture V, P or T. Growth was then assessed by tritiated thymidine uptake. Values are expressed as means ($n=6$) \pm sd.



b) Dose Response Experiments of Prostate Cell Lines To Osteoblast-like Cell Conditioned Medium

To investigate further the stimulatory activity of OBCM on prostate cancer cell line (DU145, PC-3 and LNCaP) growth, the effect of increasing dose was examined. Medium conditioned by the osteoblast culture, U, was serially diluted with SFM and each cell line was exposed to all dilutions for a period of 3 days.

The growth of both DU145 (figure 4a) and PC-3 cells (figure 4b) responded in a dose dependent fashion with increasing concentrations of OBCM. DU145 cell growth was increasingly stimulated as the concentration of OBCM increased from 0 to 6.25%, greater concentrations caused little further increase. However, only concentrations of 6.25% and above significantly ($p<0.025$) stimulated DU145 growth, compared to growth in serum free medium. Similarly, PC-3 cell growth was increasingly stimulated by concentrations of OBCM from 0 to 12.5%, with higher concentrations producing no further stimulation. The growth stimulation of PC-3 cells was only significant at concentrations of 12.5% and above ($p<0.05$).

In addition to studies with hormone insensitive cell lines, the effect of OBCM on the androgen sensitive cell line, LNCaP, was studied. Figure 4c illustrates that 3% OBCM had a slight inhibitory effect on LNCaP growth, though this wasn't significant. Increasing doses of OBCM did not affect growth until at concentrations of 12.5% and greater, growth was significantly ($p<0.05$) stimulated. All stimulatory doses produced a 1.3 fold increase of growth compared to control levels.

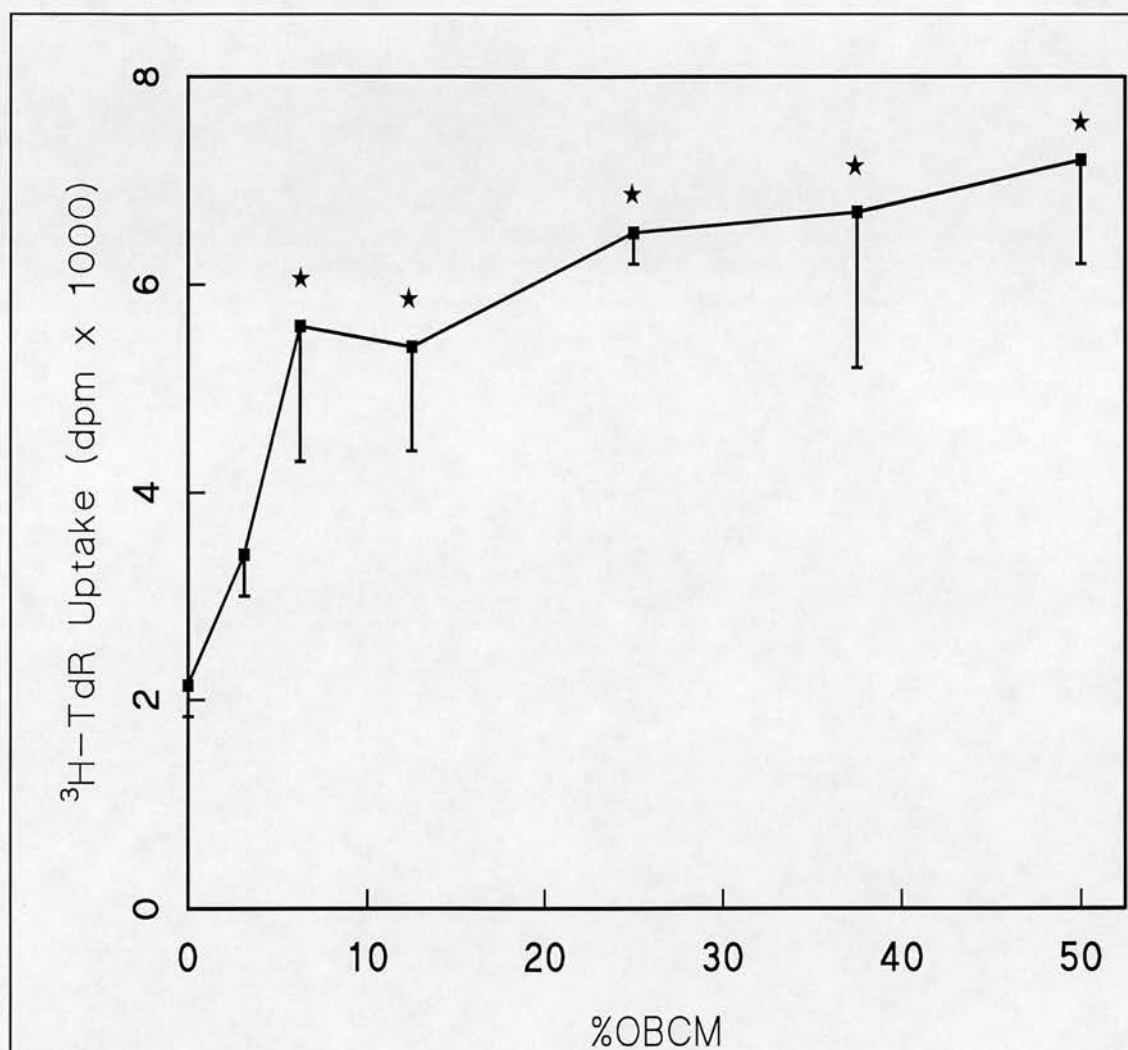


Figure 4a: Dose Response of DU145 Cells To Osteoblast-like Cell Conditioned Medium. DU145 cells were plated (1000/well) and grown in SFM. Cells were subsequently incubated in serial dilutions of OBCM (diluted with SFM) or a control of SFM, for a period of three days after which growth was measured by tritiated thymidine uptake. Values are expressed as means ($n=6$) \pm sd. p values are indicated if growth in OBCM was significantly different to growth in SFM. * $p<0.025$

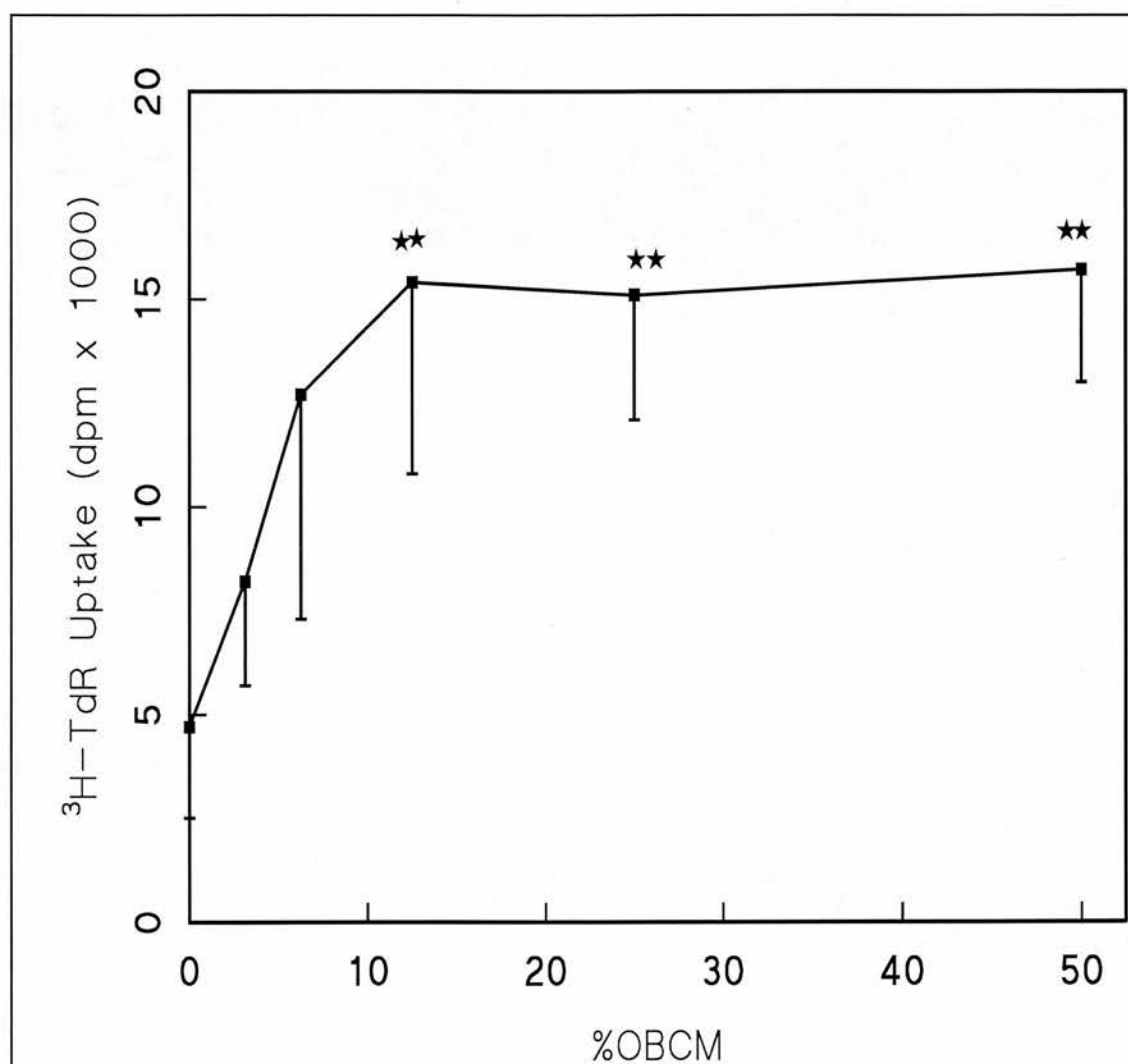


Figure 4b: Dose Response of PC-3 Cells To Osteoblast-like Cell Conditioned Medium. PC-3 cells were plated (1000/well) and then grown in SFM. Cells were subsequently incubated in serial dilutions of OBCM (diluted with SFM) or a control of SFM, for a period of three days after which growth was measured by tritiated thymidine uptake. Values are expressed as means ($n=6$) \pm sd. p values are indicated if growth in OBCM was significantly different to growth in SFM. ** $p<0.05$

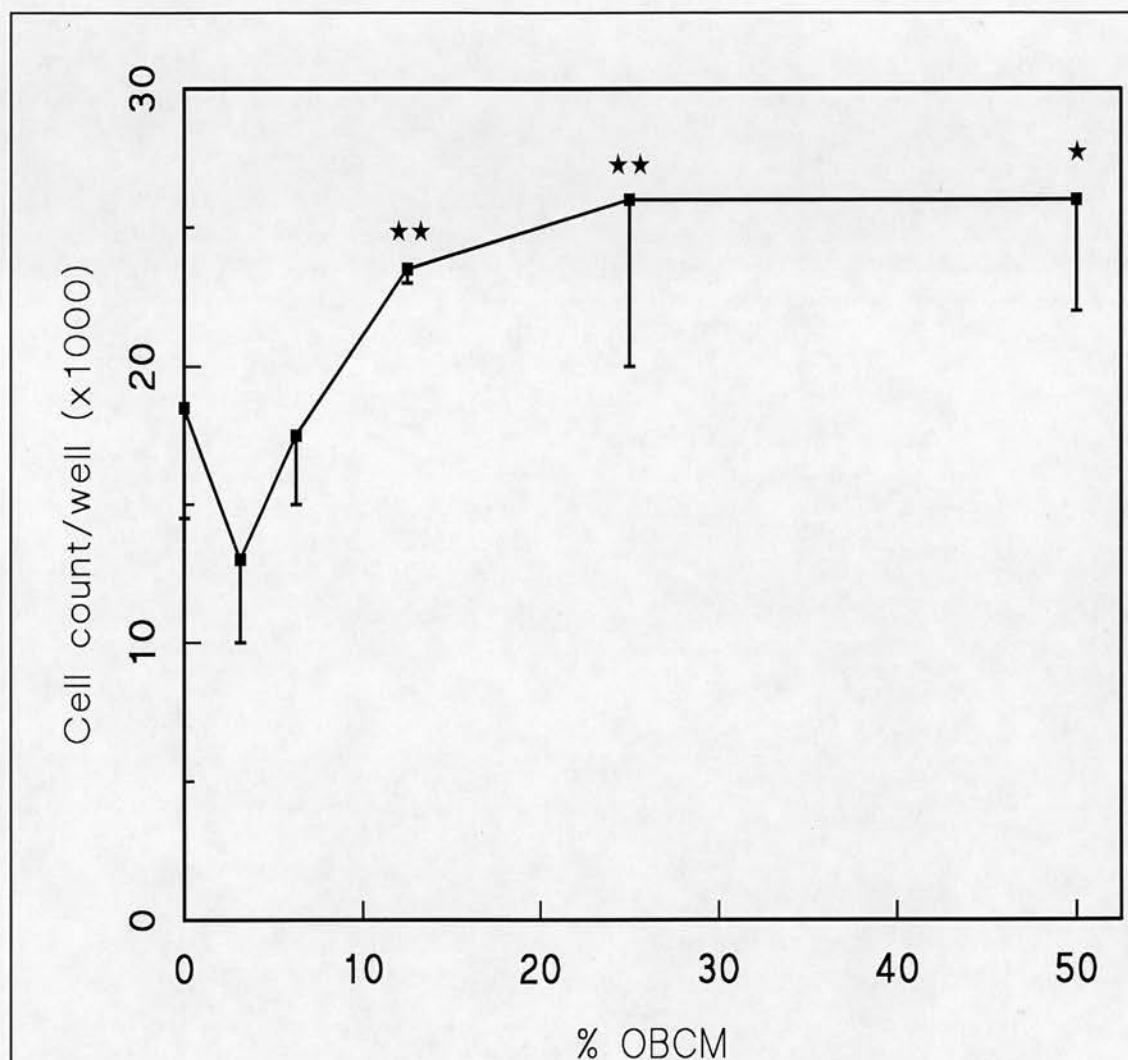


Figure 4c: Dose Response of LNCaP Cells To Osteoblast-like Cell Conditioned Medium. LNCaP cells were plated (5000/well) in normal growth medium. After 48 hours the cells were incubated in serial dilutions of OBCM (diluted with normal growth medium) or a control of normal growth medium, for a period of five days after which growth was measured by cell counts. Values are expressed as means ($n=3$) \pm sd. p values are indicated if growth in OBCM was significantly different to growth in SFM. * $p<0.025$, ** $p<0.05$

The results presented in figures 4a, 4b and 4c are typical of the dose dependent effects observed when prostate cell lines are incubated with media conditioned by other osteoblast-like cell cultures. A further example, using media conditioned by the osteoblast culture, P, is summarised in table 2. These results indicate that in a manner similar to 'OBCM (U)', 'OBCM (P)' stimulated PC-3 and DU145 cell growth in a dose dependent manner and LNCaP cells were stimulated only by high doses of OBCM. Again, it is evident that the magnitude of stimulation induced by conditioned media can vary with different osteoblast cultures, but the overall pattern between the cell lines remains the same - PC-3 cells were always stimulated to the greatest extent (3-3.6 fold), followed by DU145 cells (2.1-3.3 fold) and finally LNCaP cells (1.3-1.5 fold).

Table 2: Dose Response of Prostate Cell Lines To Osteoblast-like Cell Conditioned Medium (P).

Cells were plated and exposed to 'OBCM (P)' as described in the legends for figures 4a, 4b and 4c. Values in bold indicate the mean growth stimulation as a multiple of growth in OBCM/growth in control. Within the brackets are the mean value (n=6) of dpm (DU145 and PC-3) or cell counts/well (for LNCaP) \pm sd. p values are indicated if growth in OBCM was significantly different to growth in the control.

% OBCM (P)	PC-3 Growth Stimulation CM/Control (dpm \pm sd)	DU145 Growth Stimulation CM/Control (dpm \pm sd)	LNCaP Growth Stimulation CM/Control (cells/well \pm sd)
0	0 (1500 \pm 145)	0 (2180 \pm 210)	0 (15000 \pm 1500)
3.125	1.7 (2500 \pm 190) p<0.0025	1.38 (3040 \pm 500)	1.07 (16000 \pm 2000)
6.25	2.6 (3900 \pm 400) p<0.0005	1.67 (3680 \pm 195) p<0.0025	0 (15000 \pm 2000)
12	2.8 (4250 \pm 200) p<0.0005	1.75 (3850 \pm 370) p<0.0025	1.33 (20000 \pm 1000) p<0.01
25	3.3 (5000 \pm 450) p<0.0005	1.85 (4050 \pm 400) p<0.0005	1.53 (23000 \pm 1500) p<0.0025
50	3.6 (5500 \pm 550) p<0.0005	2.1 (4590 \pm 430) p<0.0005	1.53 (23000 \pm 2000) p<0.0025

c) The Effects of Time On DU145 and PC-3 Growth In Osteoblast-like Cell Conditioned Medium

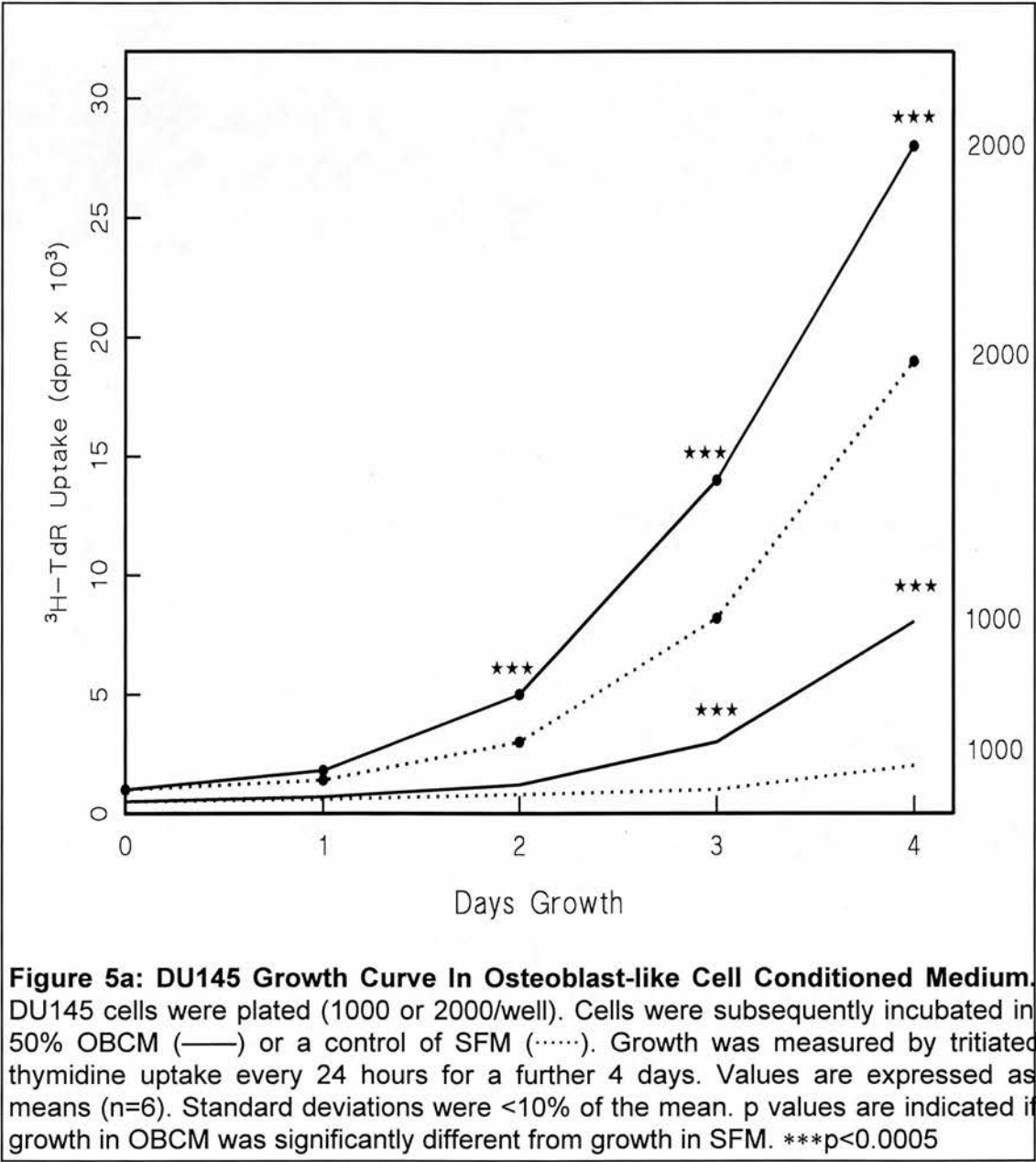
The effect of time on the OBCM stimulation of DU145 and PC-3 cell growth was studied by incubating both cell lines in 50% 'OBCM (R)' and measuring growth every 24 hours for a period of 4 days.

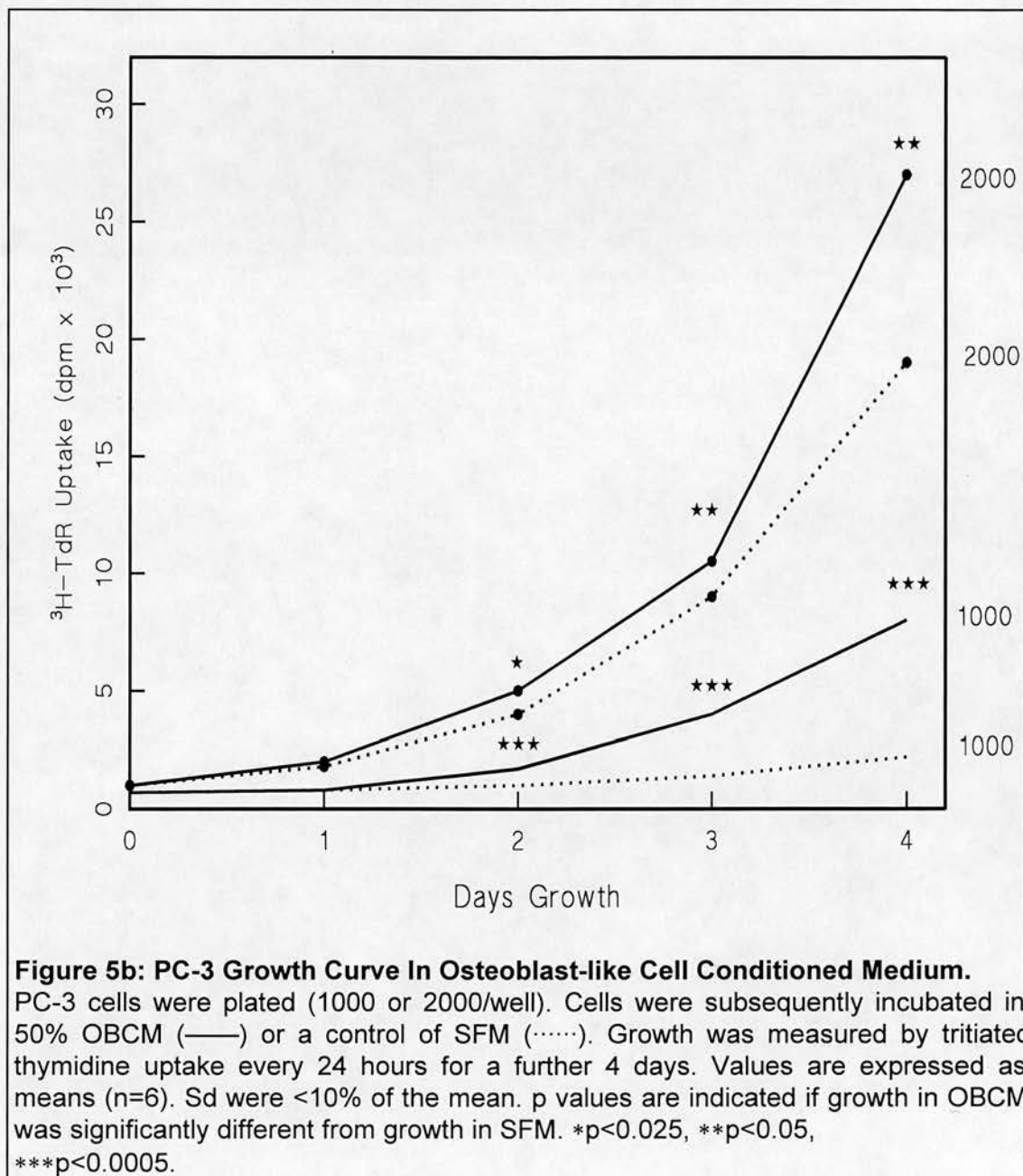
Figure 5a shows that DU145 growth steadily increased over 4 days in both serum free medium and OBCM. When the cells were plated at a density of 1000 cells/well the growth in OBCM was greater than that in serum free medium on all days and this stimulation became significant on day 3 and 4 ($p < 0.0005$). Similar results were found if the cells were plated at 2000 cells/well. At this density stimulation became significant on day 2 and thereafter ($p < 0.0005$).

PC-3 cell growth (figure 5b) was also stimulated by OBCM (in comparison to growth in SFM) over the 4 day period whether plated at 1000 or 2000 cells/well. Growth stimulation became significant (p values indicated on the graph) on day 2 and thereafter at both plating densities.

d) The Effects of Time On Prostate Cell Lines Exposed to Human Skin Fibroblast Cell Conditioned Medium

To determine if the growth stimulation of prostate cancer cell lines induced by OBCM was specific to osteoblastic mitogens, the growth effects of media conditioned by human, primary skin fibroblasts was investigated. Skin fibroblast CM was diluted to 50% with SFM and exposed to prostate cell lines. This dilution of skin fibroblast CM was used since previous experiments with OBCM showed that this concentration of CM could significantly stimulate prostate cell lines. Growth was subsequently assessed every 24 hours for 4 days.





Both DU145 (figure 6a) and PC-3 cells (figure 6b) showed increased growth with increasing time in culture, however there was no difference in the growth of the cells whether cultured in 50% skin fibroblast CM or serum free medium.

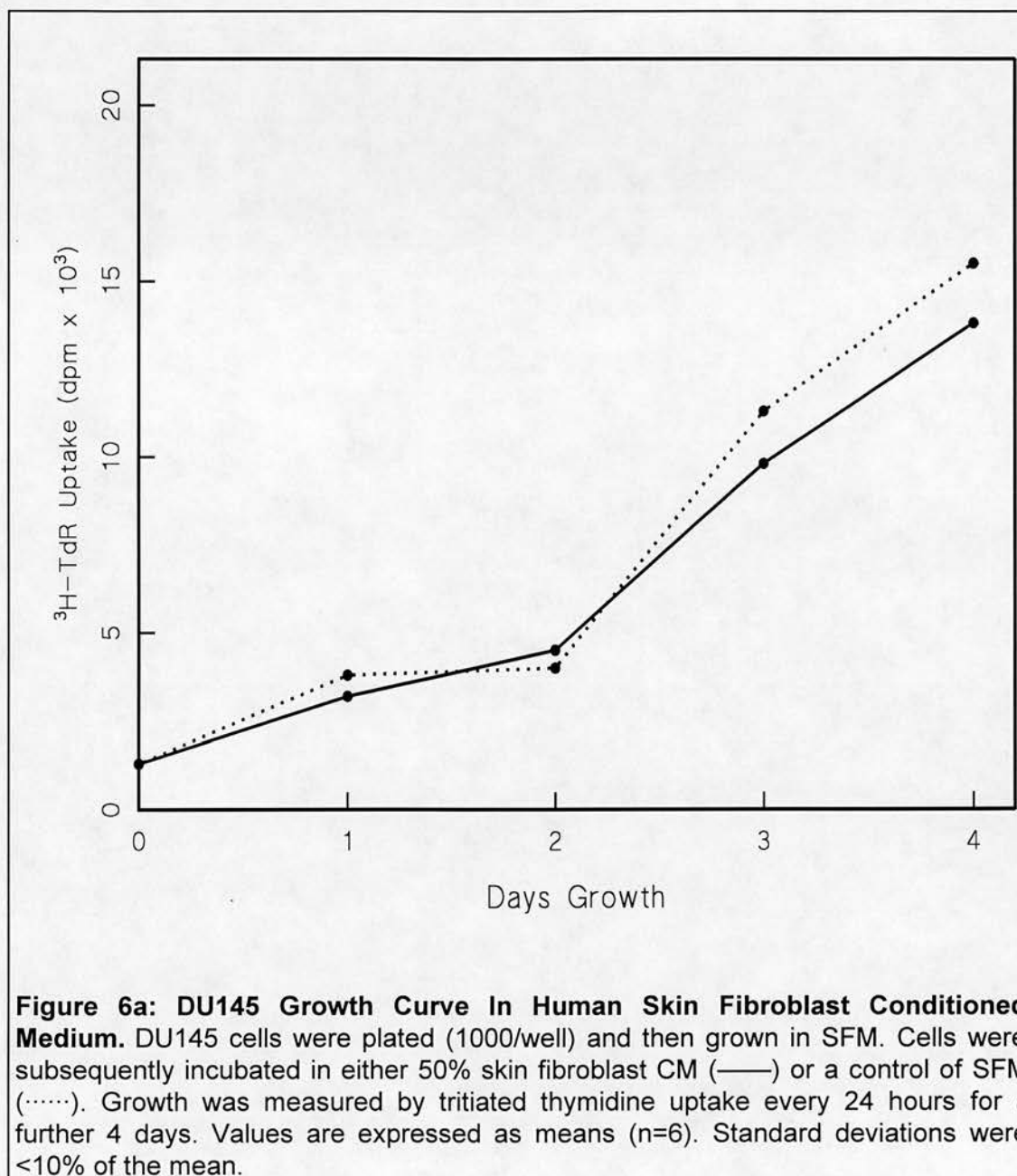
These results indicate that although the growth of DU145 and PC-3 cells was stimulated by 50% OBCM, growth was unaffected by 50% skin fibroblast conditioned medium.

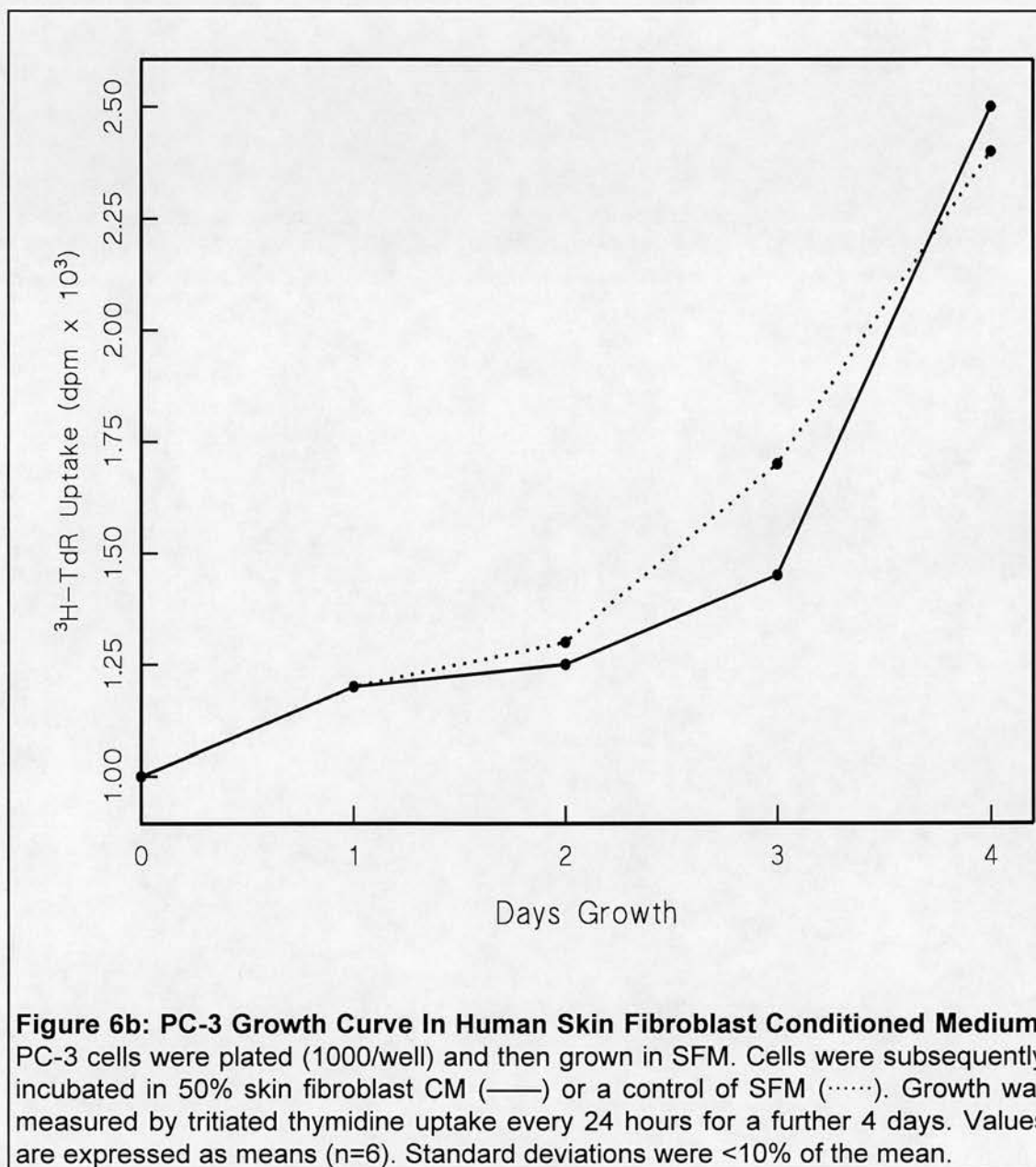
e) The Effect Of Conditioned Media From A Variety Of Cell Lines On DU145 and PC-3 Cell Growth

The specificity of osteoblast-derived factors to stimulate prostate cancer cell line growth, was investigated further by analysing the effects of cells from other potential, prostate metastatic sites. Media conditioned by cell lines derived from bladder (RT112), lung (HF19) and kidney (ACHN) carcinomas were collected and diluted to 50% with serum free medium. The effect of exposing DU145 (figure 7a) or PC-3 (figure 7b) cells to each of these media, for 3 days was assessed.

Media conditioned by bladder, lung, and kidney cell lines showed no significant stimulatory effects on DU145 (figure 7a) or PC-3 cell growth (figure 7b) when compared to growth in serum free medium. In the same experiments, the growth of both DU145 and PC-3 cells was significantly ($p < 0.0005$) stimulated by 50% 'OBCM (Q)'. The growth of DU145 and PC-3 cells in OBCM increased to 2.3 and 3.3 fold (respectively) the levels in SFM.

These results are typical of those produced on two other occasions (summarised in table 3), and together indicate that 50% CM from bladder, lung and kidney cell lines had no significant effect on PC-3 or DU145 cell growth.





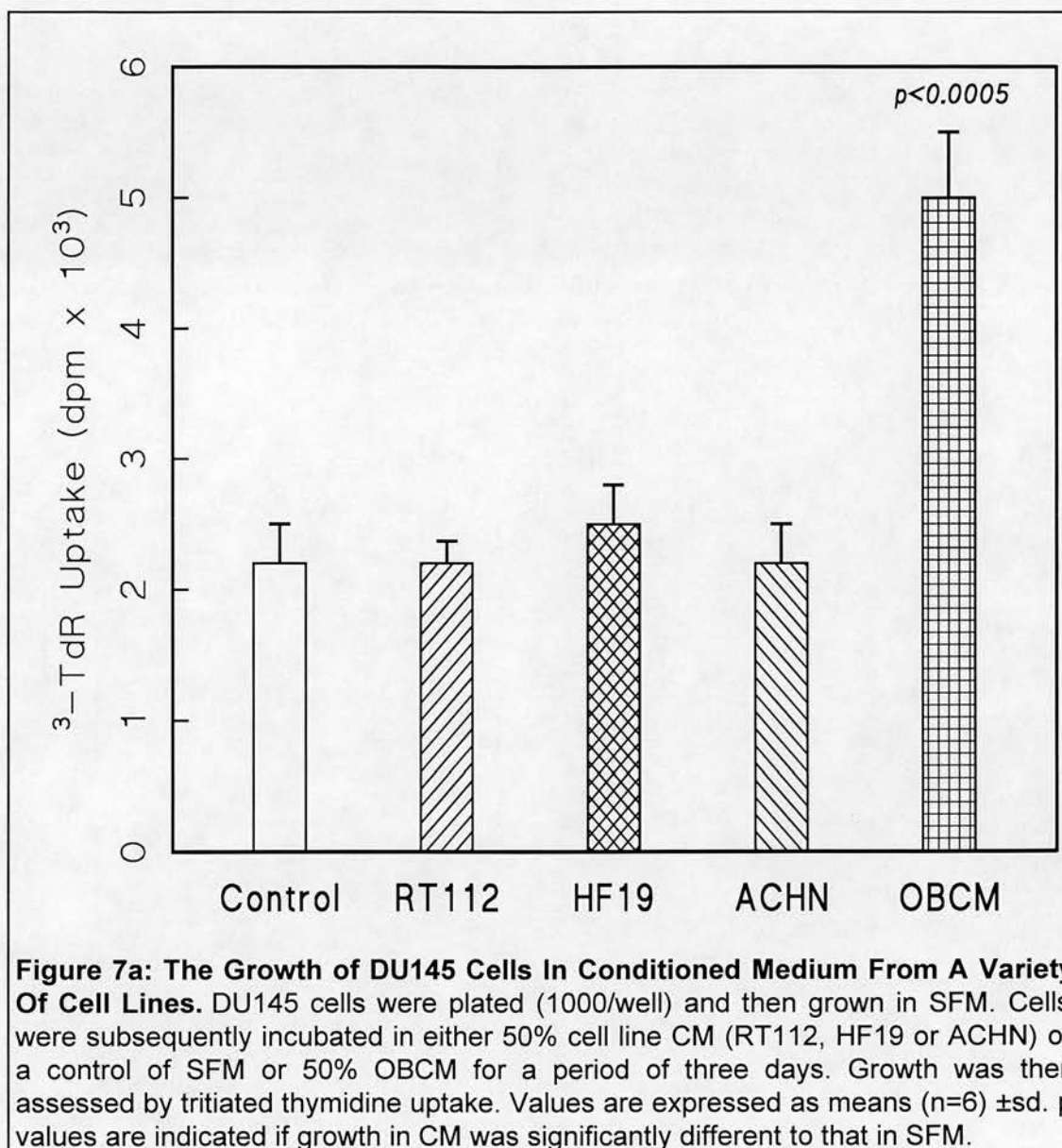


Figure 7a: The Growth of DU145 Cells In Conditioned Medium From A Variety Of Cell Lines. DU145 cells were plated (1000/well) and then grown in SFM. Cells were subsequently incubated in either 50% cell line CM (RT112, HF19 or ACHN) or a control of SFM or 50% OBCM for a period of three days. Growth was then assessed by tritiated thymidine uptake. Values are expressed as means ($n=6$) \pm sd. p values are indicated if growth in CM was significantly different to that in SFM.

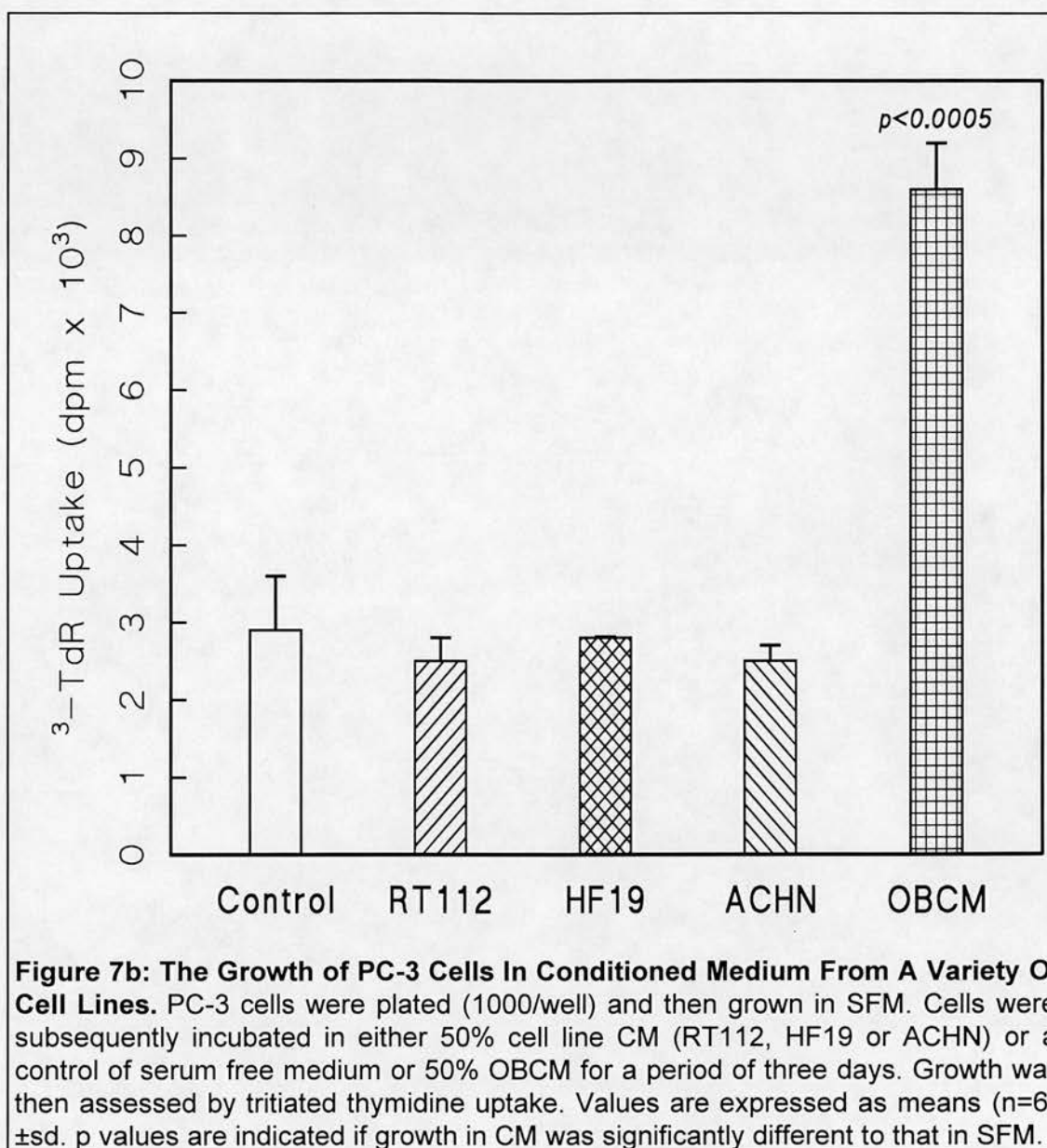


Table 3: The Growth of Prostate Cell Lines In Conditioned Medium From A Variety Of Cell Lines.

Cells were plated and exposed to 50% cell line CM as described in the legends for figures 7a and 7b. Values are expressed as means (n=6) and sd were <15% of the mean. The magnitude of either stimulation or inhibition (growth in CM/growth in SFM) is shown in brackets.

Cell Line	SFM dpm	RT112 CM dpm	HF19 CM dpm	ACHN CM dpm
DU145	1900	1750 (0.92)	1800 (0.95)	2100 (1.10)
DU145	2100	2100 (0.00)	2000 (0.95)	2150 (1.02)
PC-3	1600	1750 (1.09)	1550 (0.97)	1500 (0.94)
PC-3	2500	2550 (1.02)	2400 (0.96)	2350 (0.94)

f) The effect of osteoblast-like cell CM on liver, bladder and breast cell lines.

OBCM was assessed for its ability to stimulate cancers, other than of prostate origin, which are known to metastasise to bone. 50% 'OBCM (L)' was exposed to cell lines derived from human liver epithelial cells (CHANG), bladder epithelial carcinoma (RT112) or breast epithelial carcinosarcoma (Hs578T) cell lines. Growth was measured, after 3 days exposure to OBCM by tritiated thymidine uptake. Figure 8 demonstrates that 50% 'OBCM (L)' had no significant effects on CHANG, RT112 or Hs578T cell growth, whilst still producing a 1.7 fold increase in DU145 cell growth (in comparison to SFM). This experiment was reproduced using CM from a different osteoblast culture (U). The results obtained are summarised in table 4. These demonstrated that the growth of CHANG, RT112 and Hs578T cells were again unaffected by incubation in an OBCM which, by contrast, had stimulatory effects on DU145 cell growth.

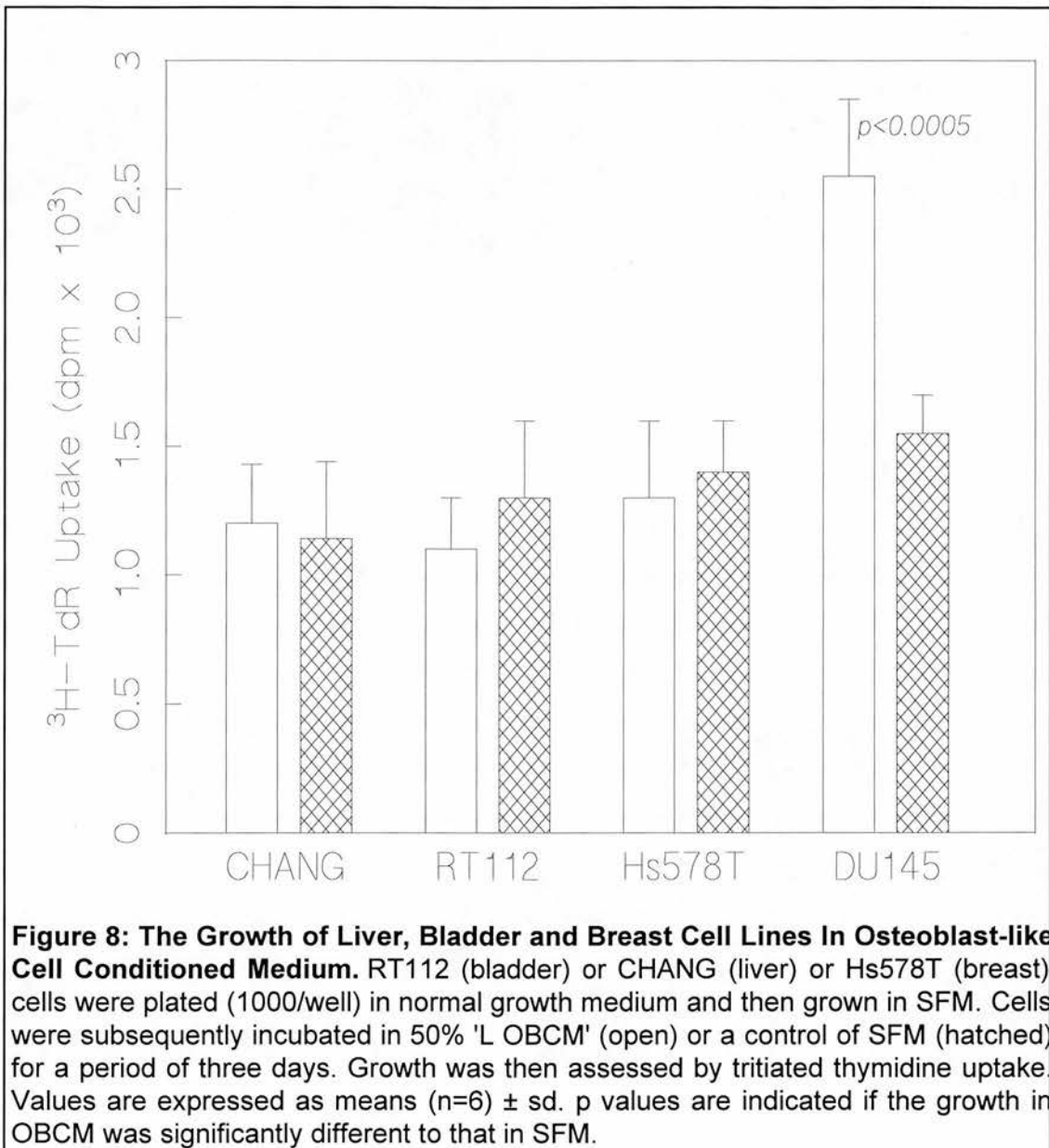


Table 4: The Growth of Liver, Bladder and Breast Cell Lines In Osteoblast-like Cell Conditioned Medium.

RT112 (bladder) or CHANG (liver) or Hs578T (breast) cells were plated and exposed to 50% 'OBCM (U)' as described in the legend for figure 8. Values are expressed as the mean of six samples \pm sd.

Cell Line	50% U OBCM (dpm \pm sd)	Control (dpm \pm sd)
RT112	1600 \pm 100	1650 \pm 190
Hs578T	1660 \pm 130	1890 \pm 200
CHANG	1010 \pm 170	1100 \pm 150
DU145	2300 \pm 260	1100 \pm 150

3.2 Characterisation of osteoblast-like cell conditioned medium

The results so far obtained have demonstrated that OBCM can stimulate the growth of prostate cell lines PC-3, DU145 and LNCaP. In contrast, red BMCM from proximal femora and skin fibroblast CM, diluted to 50% with SFM, did not stimulate the same prostate cancer cell lines. CM (also 50% dilute) from kidney, bladder or lung cell lines were also ineffective. Additionally, the growth of cell lines derived from liver, bladder and breast cancers were unaffected by incubation with 50% OBCM.

To characterise the nature of the prostate stimulatory component(s) in OBCM four studies were carried out;

- i) heating the OBCM to 80°C for one hour
- ii) incubation of OBCM with citric acid-phosphate buffers of different pH
- iii) determining the effect of 1,25 (OH)₂ vitamin D and DHT on production and stimulatory activity
- iv) molecular weight fractionation with 'centricon' filters

a) Heat Treatment of OBCM

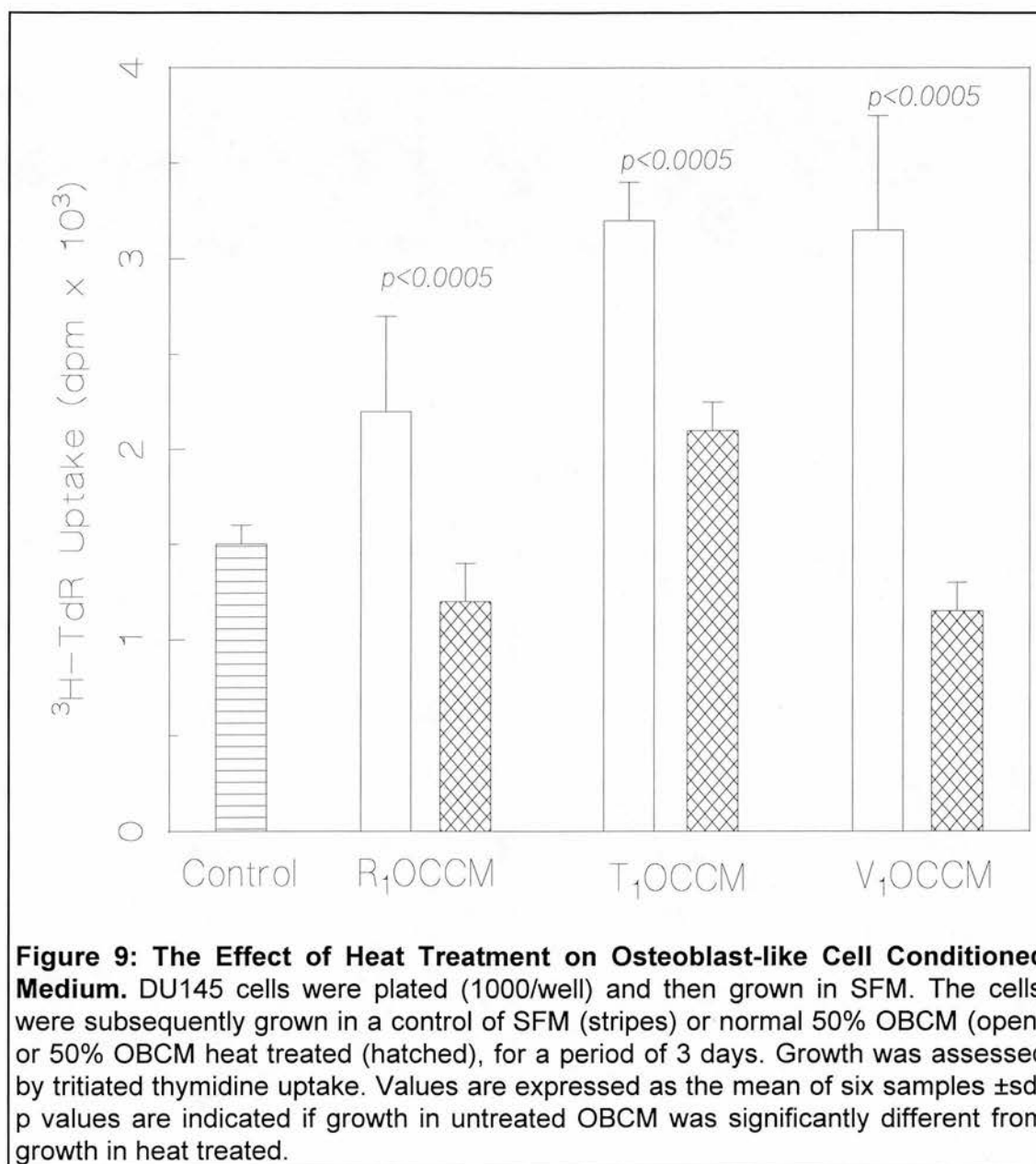
The effect of heat treatment on OBCM, was determined by heating each of three different OBCM (R, T, V) to 80°C for one hour (Mohan *et al* 1986). Their ability to stimulate DU145 growth was then assessed using thymidine uptake.

Figure 9 shows that prior to heat treatment each OBCM was capable of stimulating DU145 growth in comparison to SFM. However after heat treatment growth was partially reduced (T) or reduced to levels less than the control (R and V) . The reduction of stimulation was significant ($p < 0.0005$) in each case.

b) Acid Treatment of OBCM

The effect of low pH on OBCM was judged by incubating U OBCM in citric acid-phosphate buffers of different acidities (pH 2.6, 4.6, 6.6 and 7.6) for one hour. The pH was then returned to 7.6 with BSA solution and the ability of each solution to stimulate DU145 growth was again assessed with thymidine uptake.

The results shown in figure 10 indicate that OBCM treated with a buffer of pH 7.6 or 6.6 retain their stimulatory activity over DU145 growth. If OBCM was treated with a buffer of pH 4.6 or 2.6 the stimulatory activity was lost and growth became lower than control levels.



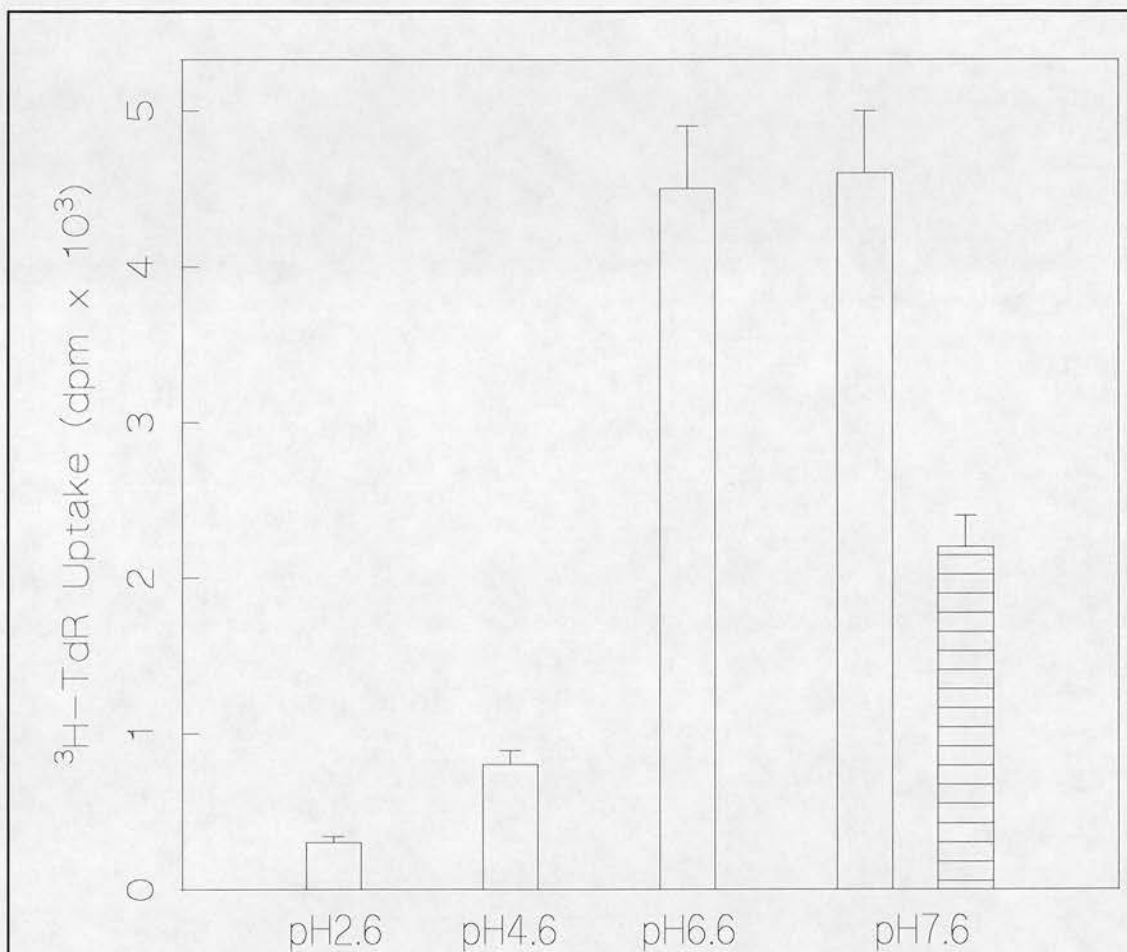


Figure 10: The Effect of Acidic Buffers on Osteoblast-like Cell Conditioned Medium. DU145 cells were plated (1000/well) and then grown in SFM. Cells were subsequently grown in a control of SFM (stripes) or 50% OBCM (open) treated with a citric acid-phosphate buffer (pH 2.6-7.6), for a period of 3 days. Growth was assessed by tritiated thymidine uptake. Values are expressed as the mean of six samples \pm sd.

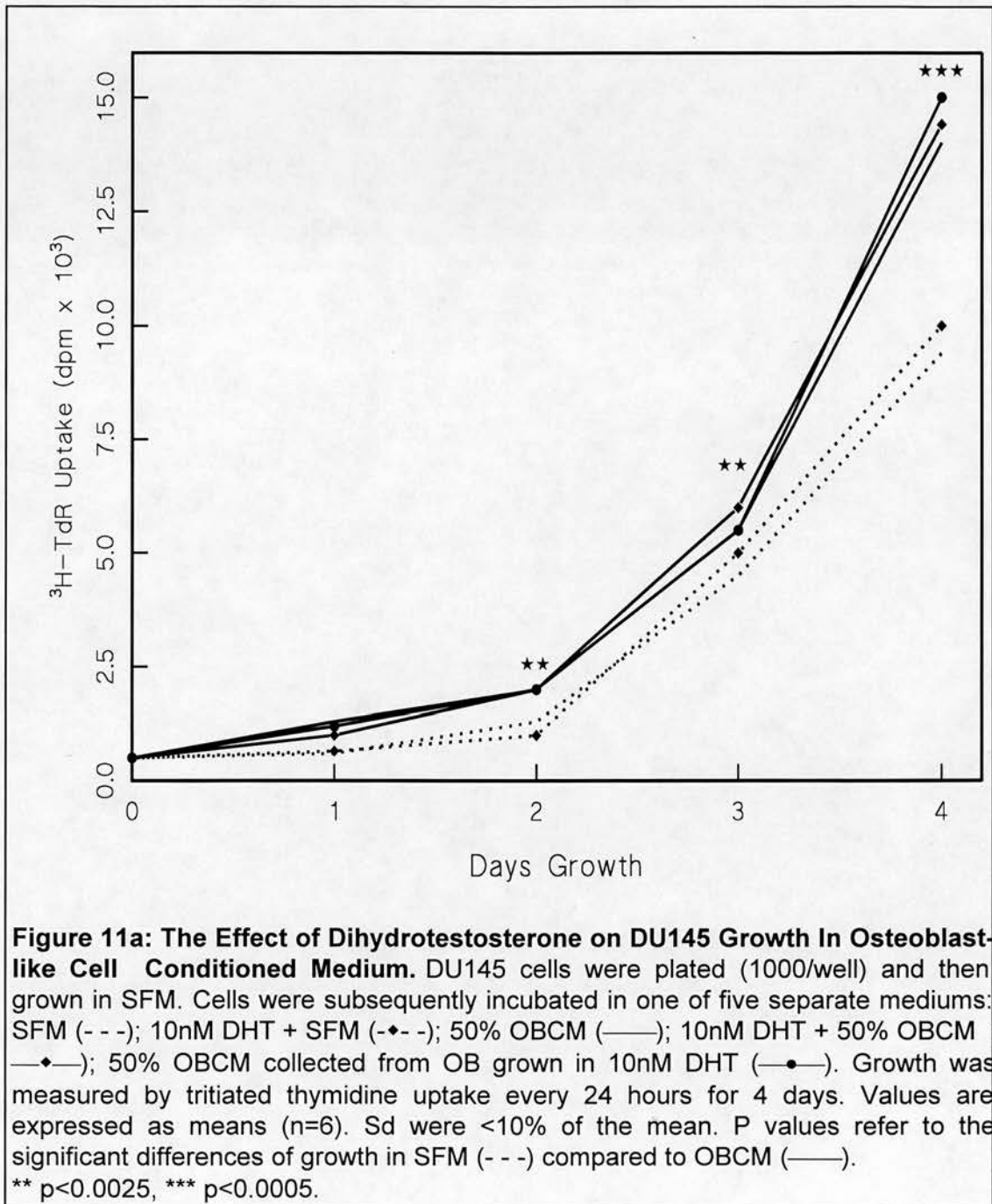
c) The effects of DHT and 1,25 (OH)₂ Vitamin D On OBCM Stimulatory Activity

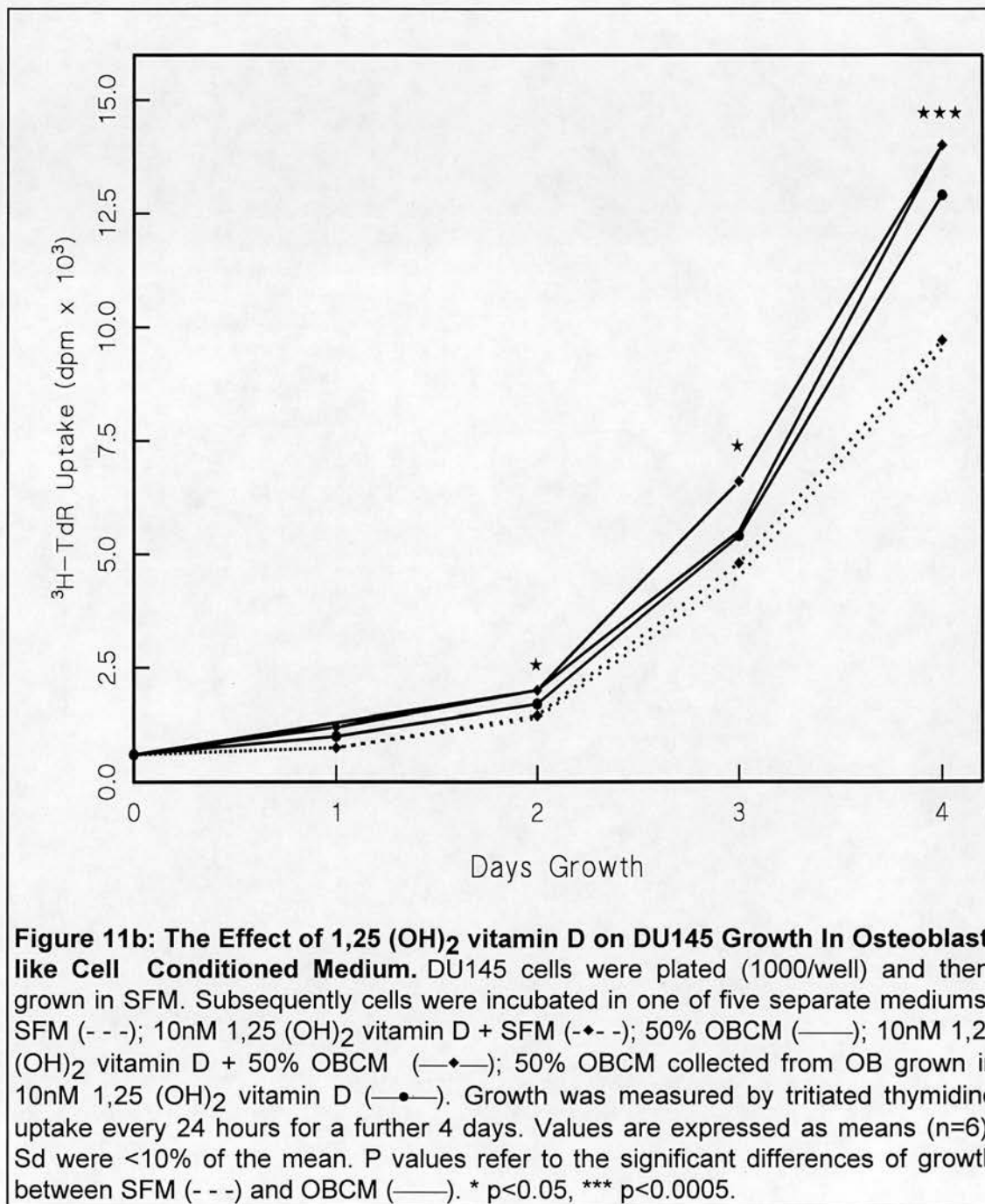
To characterise further the prostate stimulatory factors in OBCM, the effects of hormones which target either prostate or bone (DHT and 1,25 (OH)₂ vitamin D respectively) were examined. Two investigations were carried out:

- 1) To gauge the effect of adding 10nM DHT (Horoszewicz *et al* 1983) or 10nM 1,25 (OH)₂ vitamin D (Beresford *et al* 1984, Murray *et al* 1987) to OBCM, and its subsequent effect on the growth stimulation of DU145 cells.
- 2) To determine whether the growth of DU145 cells is influenced by CM collected from osteoblasts grown in the presence of either 10nM DHT or 10nM 1,25 (OH)₂ vitamin D.

Figure 11a and 11b illustrate that DU145 growth was stimulated by 50% 'OBCM (T)' in comparison to growth in SFM. Growth stimulation was significant on day 2 and thereafter (p values are indicated on the graphs). Supplementation of 50% OBCM or SFM with either 10nM DHT (figure 11a) or 10nM 1,25 (OH)₂ vitamin D (figure 11b) had no effect on the level of DU145 growth in either medium. DU145 growth was also unaffected by media conditioned by osteoblasts grown in the presence of 10nM DHT (figure 11a) or 10nM 1,25 (OH)₂ vitamin D (figure 11b).

These results are typical of those produced on other occasions, indicating that the production of DU145 stimulatory factors from osteoblast-like cells is unaffected by 10nM DHT or 10nM 1,25 (OH)₂ vitamin D. Also the addition of either hormone directly to the OBCM had no effect on the DU145 stimulatory activity.





d) Molecular Weight Fractionation of Osteoblast-like Cell Conditioned Medium

50% 'OBCM (U)' was sequentially filtered through a set of four 'centricon' filters with decreasing molecular weight pore sizes (100Kd, 30Kd, 10Kd and 3Kd) to crudely establish the molecular weights of prostate mitogens in OBCM. The filters produced five molecular weight fractions of OBCM; '>100Kd', '<100Kd', '<30Kd', '<10Kd' and '<3Kd'. Each of these fractions was tested for its ability to stimulate DU145 growth. SFM was similarly filtered to provide five control fractions.

Typical results are shown in figure 12. Unfractionated OBCM stimulated DU145 growth 2.7 fold ($p<0.0005$) compared to unfractionated SFM. The level of this stimulation decreased only slightly when the cells were incubated in the fraction '>100Kd'. However stimulatory activity significantly ($p<0.0005$) decreased to 1.8 fold when the cells were incubated in the fraction '<100Kd'. DU145 cells grown in the fraction '<30Kd' showed no growth stimulation above control levels. This too was a significant ($p<0.05$) decrease in stimulatory activity compared to unfractionated OBCM or the fraction '<100Kd'. The fractions of OBCM '<10Kd' and '<3Kd' both had no stimulatory effects on DU145 cell growth.

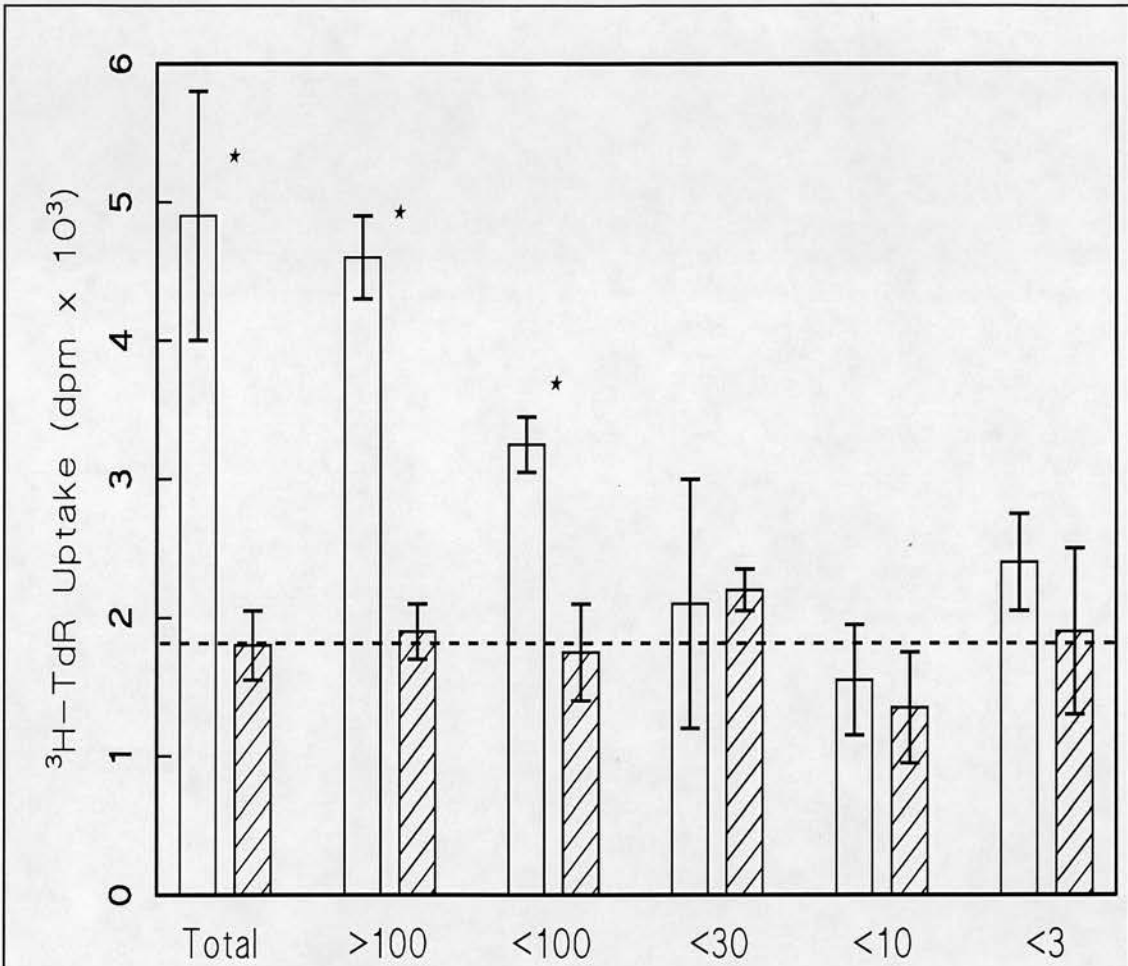


Figure 12: Molecular Weight Fractionation Of Osteoblast-like Cell Conditioned Medium. DU145 cells were plated (1000/well) and then grown in SFM. Cells were subsequently incubated in a control of SFM (diagonals) or 50% OBCM (open), for a period of 3 days. The media were either unfractionated (total) or had been sequentially passed through a series of filters with decreasing molecular weight cut-off points (100Kd, 30Kd, 10Kd and 3Kd). Growth was assessed by tritiated thymidine uptake. Values are expressed as the mean value (n=6) \pm sd. p values are indicated if the growth of DU145 cells in the OBCM fraction was significantly different to growth in the SFM fraction. * p<0.0005

The precision of the filters was assessed by silver staining. A 7.5% polyacrylamide gel was run with the following 1ml samples; SFM, unfractionated 50% OBCM, '>100Kd OBCM', '<100Kd OBCM' and '<30Kd OBCM'. On completion of the run, the gel was silver stained to reveal the protein bands, shown in plate 1. It illustrates the fraction '>100Kd' contained proteins greater than 100Kd but also a few proteins less than 100Kd. The fraction '<100Kd' contained no proteins greater than this molecular weight and similarly the fraction '<30Kd' contained no proteins greater than 30Kd. This indicates that although filtrates contain no proteins greater than their molecular weight cut-off point, some of the proteins below this point were retained on the filter (plate 1).

The molecular weight fractions which had stimulatory activity for DU145 growth were '>100Kd' and '<100Kd'. These were characterised with heat and acid treatment as for unfractionated OBCM (in figures 9 and 10).

The results shown in figure 13 indicate that unfractionated OBCM significantly ($p<0.0005$) stimulated DU145 growth 2.6 fold greater than the control values. The fraction '>100Kd' significantly stimulated DU145 growth 2.3 fold and this stimulatory activity decreased to control values if the fraction was incubated at 80°C for 1 hour or incubated in a buffer at pH 4.6. In a similar manner DU145 cells were significantly stimulated 1.4 fold ($p<0.025$) by the fraction '<100Kd'. This stimulatory activity also decreased to control levels if the fraction was heat treated at 80°C or incubated in buffer at pH 4.6.

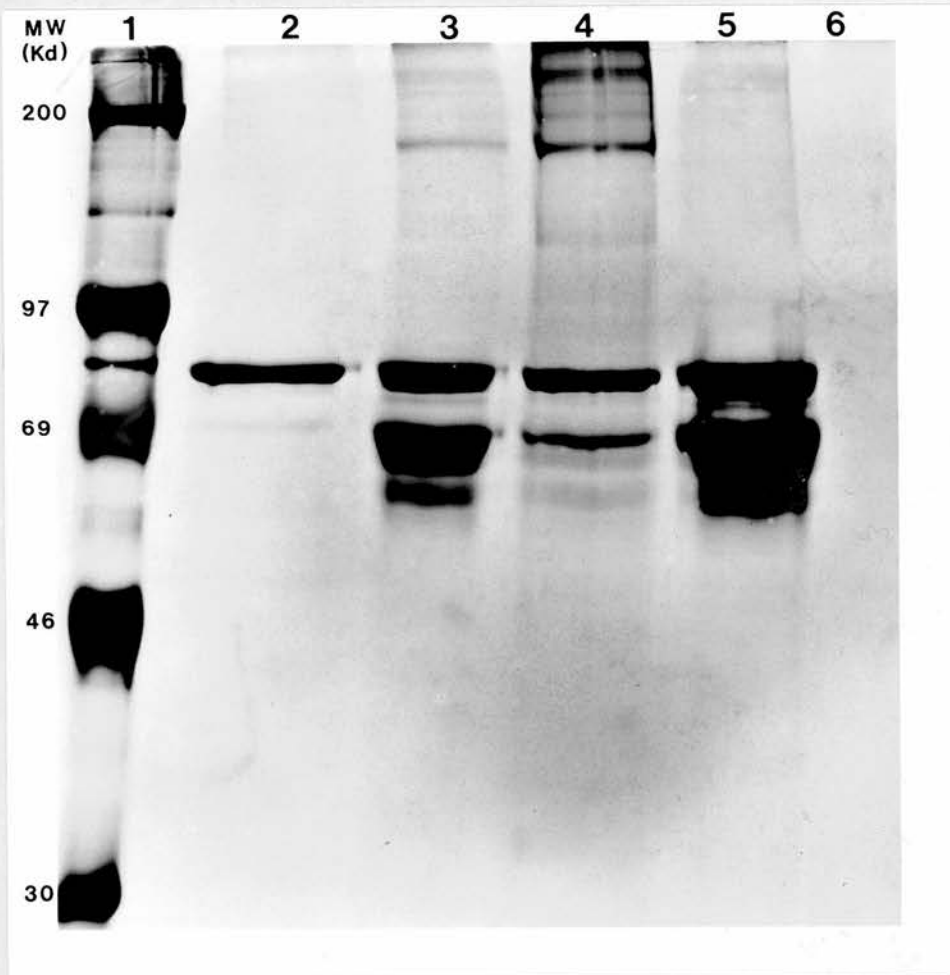
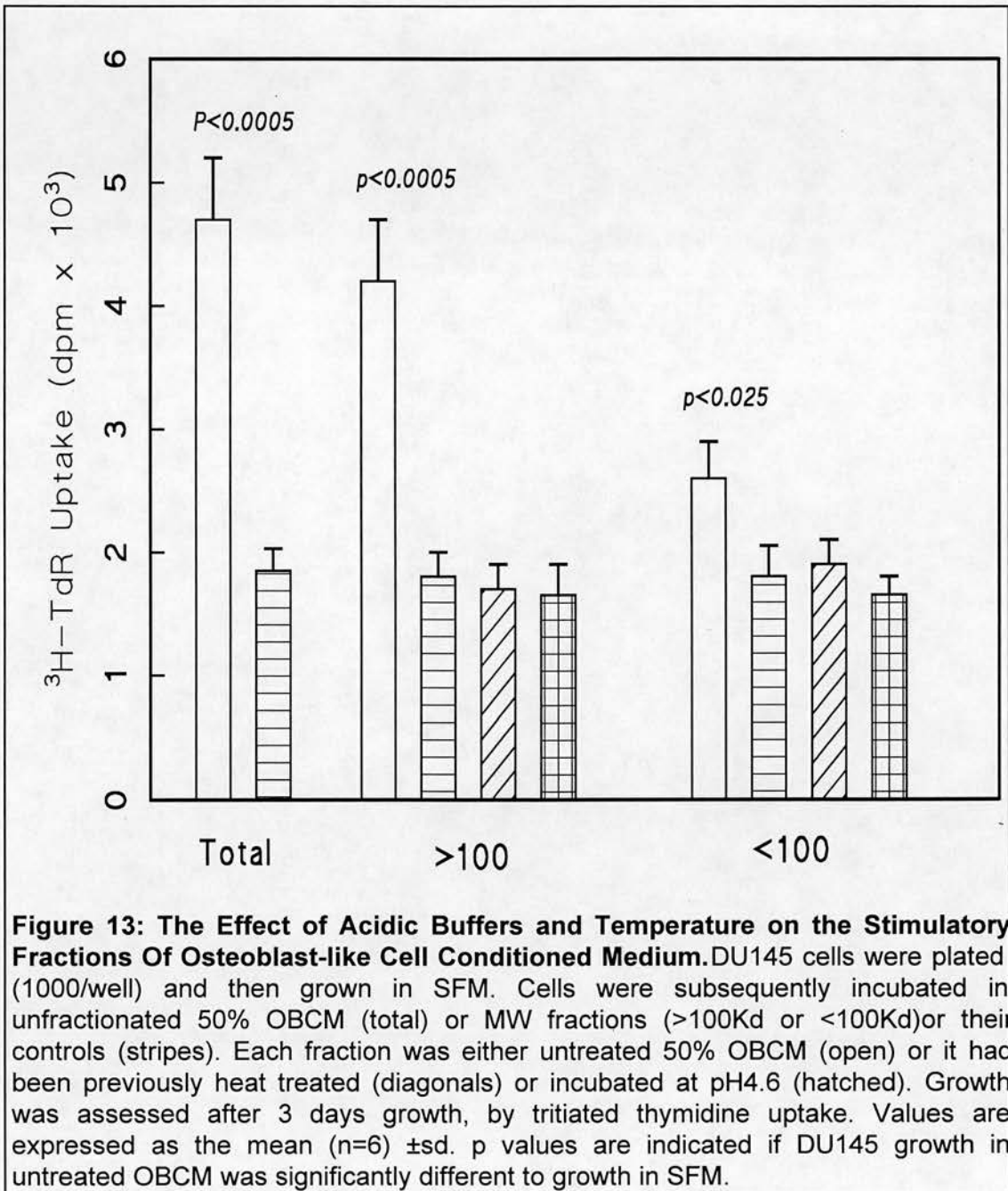


Plate 1: Silver Staining assessment of the validity of the Amicon Filters.

1ml samples of SFM (lane 2), 100% OBCM (lane 3), '>100Kd' fraction of OBCM (lane 4), '<100Kd' fraction of OBCM (lane 5), '<30Kd' fraction of OBCM (lane 6) were lyophilised to dryness and reconstituted in 50 μ l of 'blue juice' loading buffer. These were loaded on a 7.5% SDS-PAGE with molecular weight markers 14.3-200Kd (lane 1). The gel was run at 40mA for 5 hours and silver stained to identify the protein bands.



3.3 The Effects Of Haematopoietic Growth Factors On Prostate Cell Lines

The results of sections 3.1 and 3.2 have indicated that the growth of prostate cell lines can be stimulated by factors secreted from human osteoblast-like cells and not by media conditioned with either bone marrow or skin fibroblasts. To aid the identification of prostate stimulatory factors in OBCM the effect of specific growth factors found in the bone environment was tested. Haematopoietic growth factors are both produced, and have their major biological effects in the bone marrow (Nicola 1989), and two of these haematopoietic growth factors GM-CSF and G-CSF are already known to be secreted by osteoblasts (Horowitz *et al* 1989, Felix *et al* 1991). Therefore, GM-CSF and G-CSF plus two other haematopoietic growth factors, EPO and IL-3 were investigated for prostate mitogenic effects. These four haematopoietic growth factors were also of clinical interest, as they are used to revive bone marrow after chemotherapy (Oster *et al* 1990, Moore 1990). If the haematopoietic growth factors were found to stimulate the growth of prostate cancer cell lines, this may indicate detrimental effects for tumour growth *in vivo*.

a) Dose Response Experiments of Prostate Cell Line Growth To Haematopoietic Growth Factors

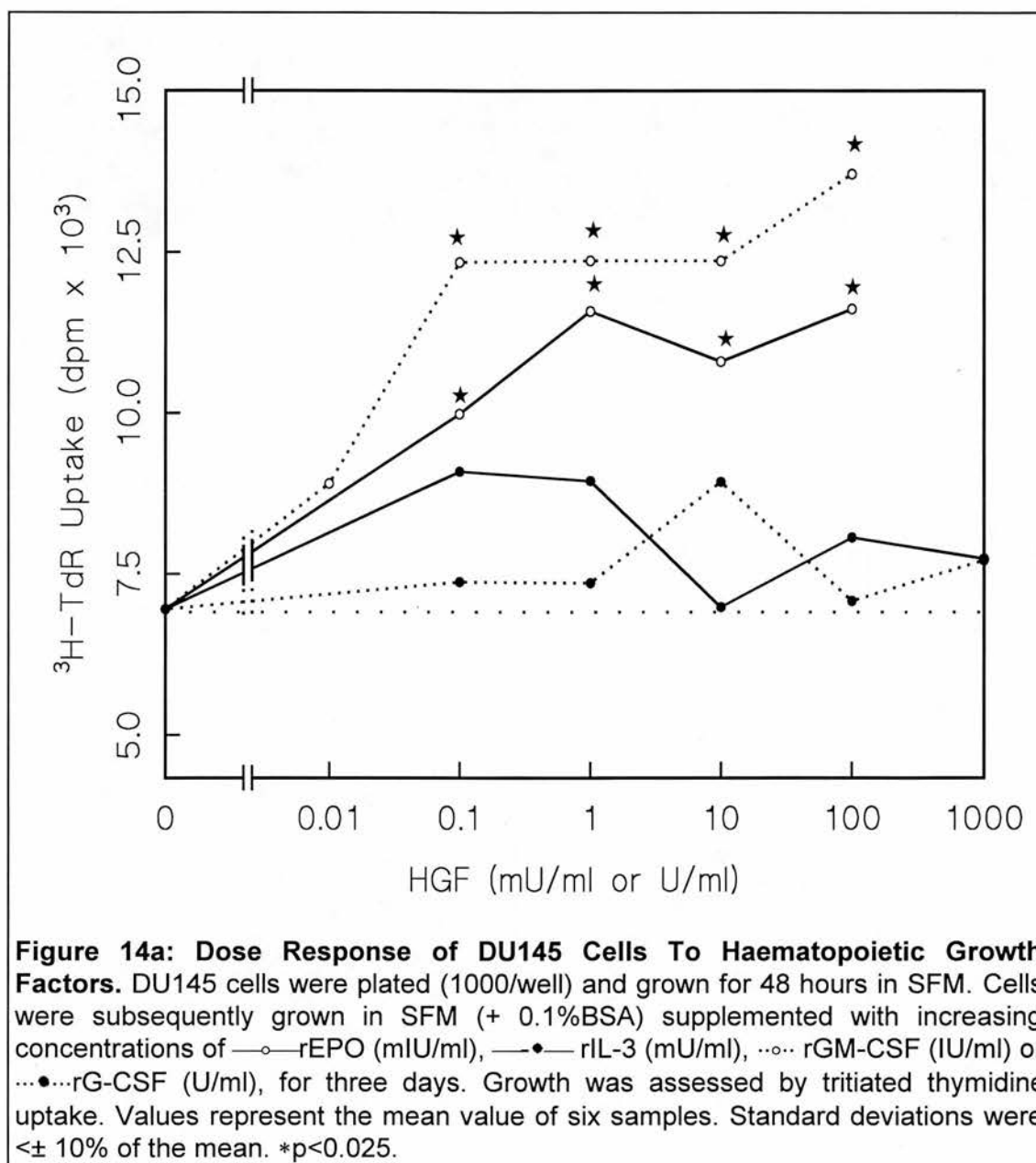
Recombinant haematopoietic growth factors were diluted to varying concentrations (based around values used clinically) with SFM plus 0.1% BSA. Prostate cell lines were then exposed to each dilution for three days, after which growth was measured by either tritiated thymidine uptake or cell counts.

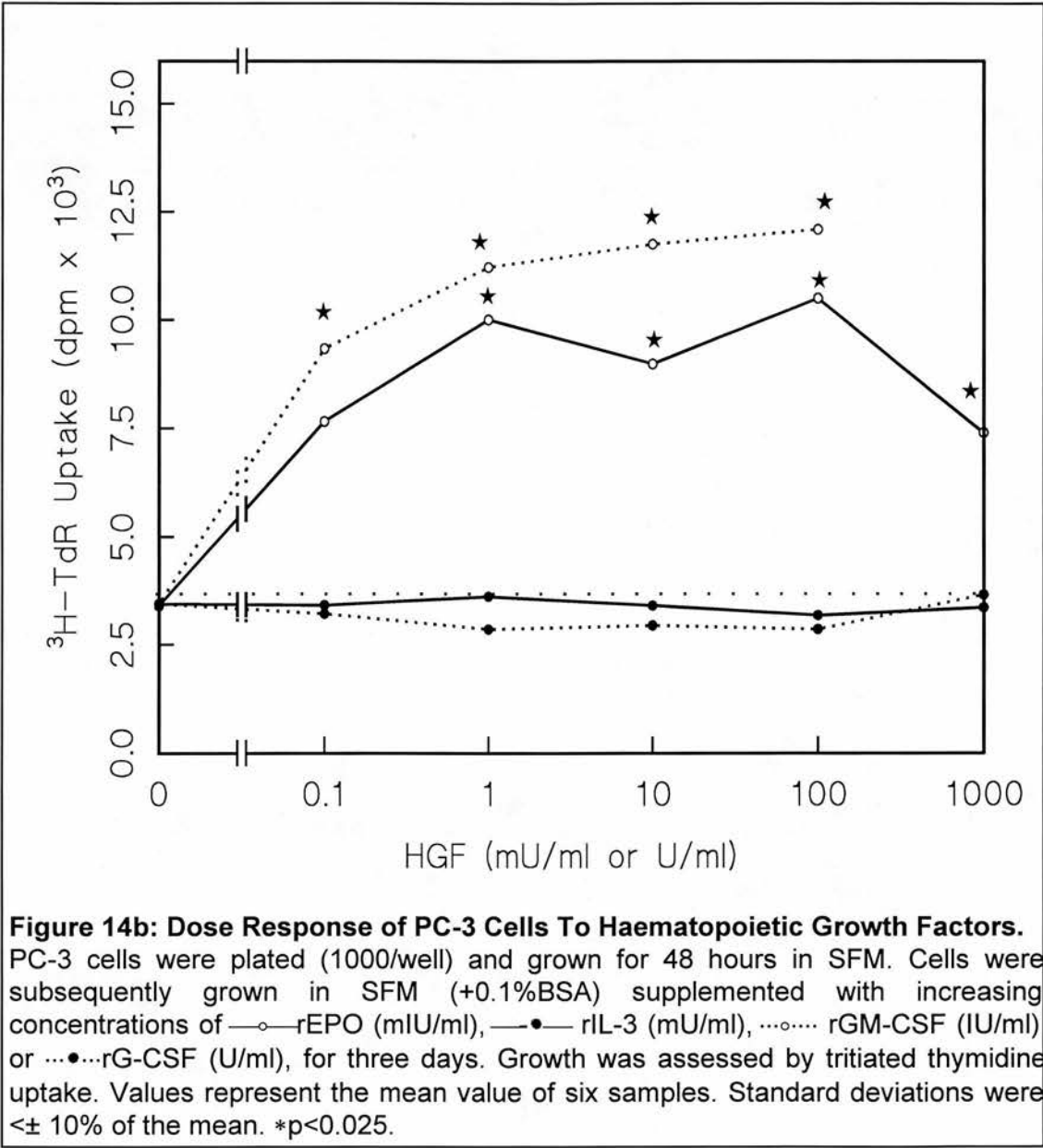
The growth of both DU145 (figure 14a) and PC-3 cells (figure 14b) showed no response to medium supplemented with either rIL-3 (0.1-1000mU/ml) or rG-CSF (0.1-1000U/ml), at any of the concentrations tested. In contrast, both DU145 and PC-3 cells responded in a dose dependent fashion to increasing concentrations of either rEPO or rGM-CSF. Increasing concentrations of rEPO up to 1mIU/ml, and concentrations of rGM-CSF up to 0.1-1IU/ml, increasingly stimulated the growth of DU145 or PC-3 cells. Concentrations greater than these caused little further increase of growth stimulation.

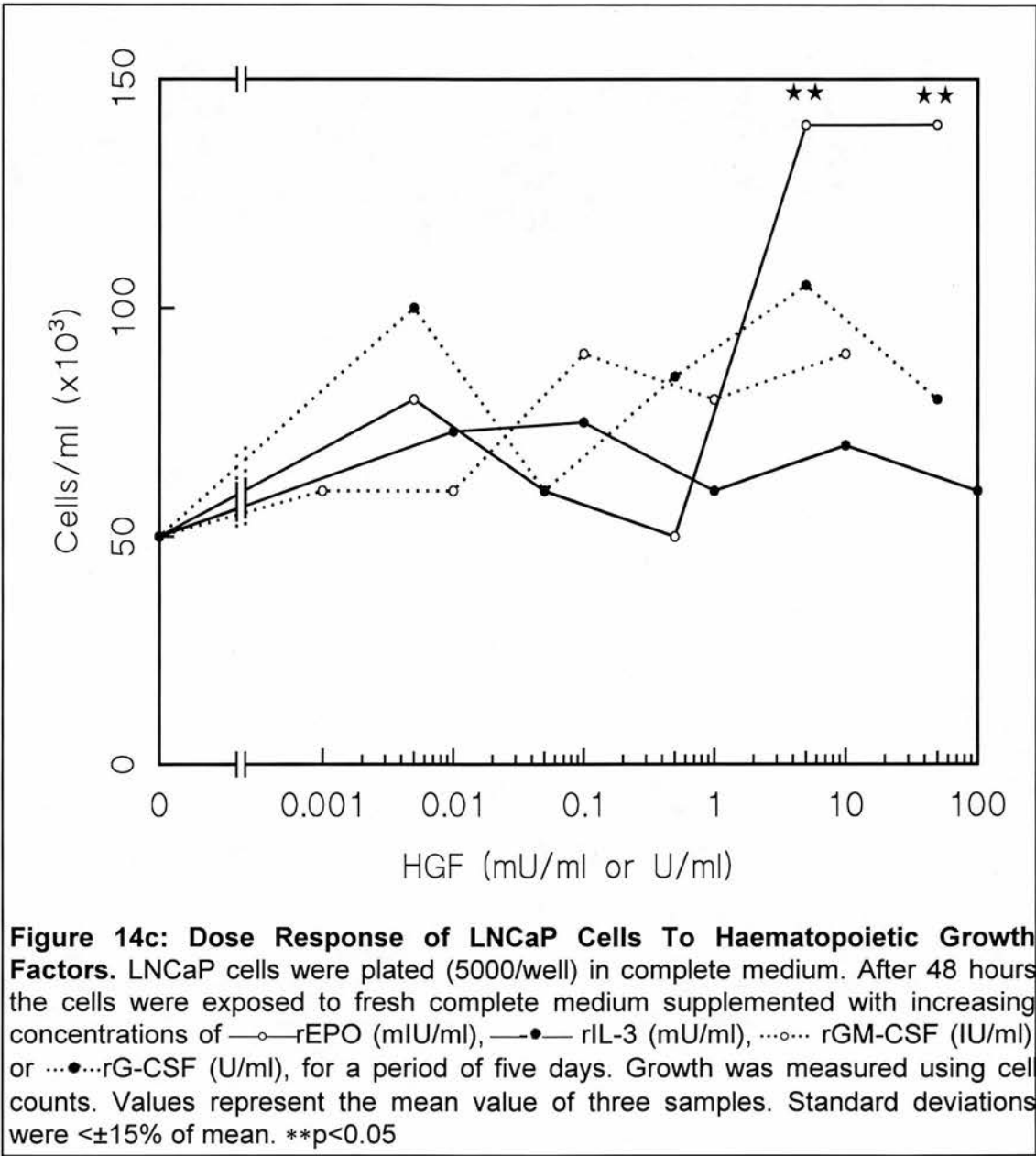
The maximum growth stimulation caused by rEPO (compared to growth in controls) was 1.7 fold for DU145 cells and 3 fold for PC-3 cells. All concentrations of rEPO significantly stimulated DU145 growth ($p<0.0025$) and concentrations ≥ 1 IU/ml significantly stimulated PC-3 cell growth ($p<0.0025$).

The maximum growth stimulation of DU145 and PC-3 cells due to rGM-CSF was 1.9 fold and 3.4 fold, respectively, in comparison to control levels. All doses of rGM-CSF, above 0.1mU/ml, significantly stimulated both PC-3 and DU145 cell growth ($p<0.0025$).

In contrast to the hormone insensitive cell lines the androgen sensitive cell line, LNCaP (figure 14c), showed no significant response to medium supplemented with rIL-3, rG-CSF or rEPO, at any of the concentrations tested. Growth was significantly stimulated by medium supplemented with rGM-CSF at doses of 5 and 50IU/ml ($p<0.05$), but not in a dose dependent fashion. Both 5 and 50 IU/ml of rGM-CSF stimulated the growth of LNCaP cells 2.8 fold compared to control levels.







The dose response effects of haematopoietic growth factors on prostate cell lines are typical of those produced on several other occasions and a further example is given in table 5.

These results again indicate that none of the three prostate cell lines responded in a significant manner to any dose of rIL-3 (0.1-1000mU/ml) or rG-CSF (0.1-1000U/ml). PC-3 and DU145 cells were again stimulated in a dose dependent fashion by both rGM-CSF (0.01-100IU/ml) and rEPO (0.01-100mIU/ml). The magnitude of PC-3 and DU145 growth stimulation, caused by rEPO (2.6 fold and 2.2 fold respectively) and rGM-CSF (3 fold and 2.3 fold respectively) was similar to the previous experiment. As before, PC-3 cells were stimulated to a greater extent than DU145 cells. LNCaP cells showed no response to rEPO, whilst growth was significantly stimulated by higher doses of rGM-CSF (≥ 10 IU/ml). In this experiment, LNCaP cells were stimulated to a maximum of 2 fold by stimulatory doses of rGM-CSF (in comparison to controls).

b) DU145 and PC-3 Growth Curve In 100mIU/ml rEPO and 100IU/ml rGM-CSF

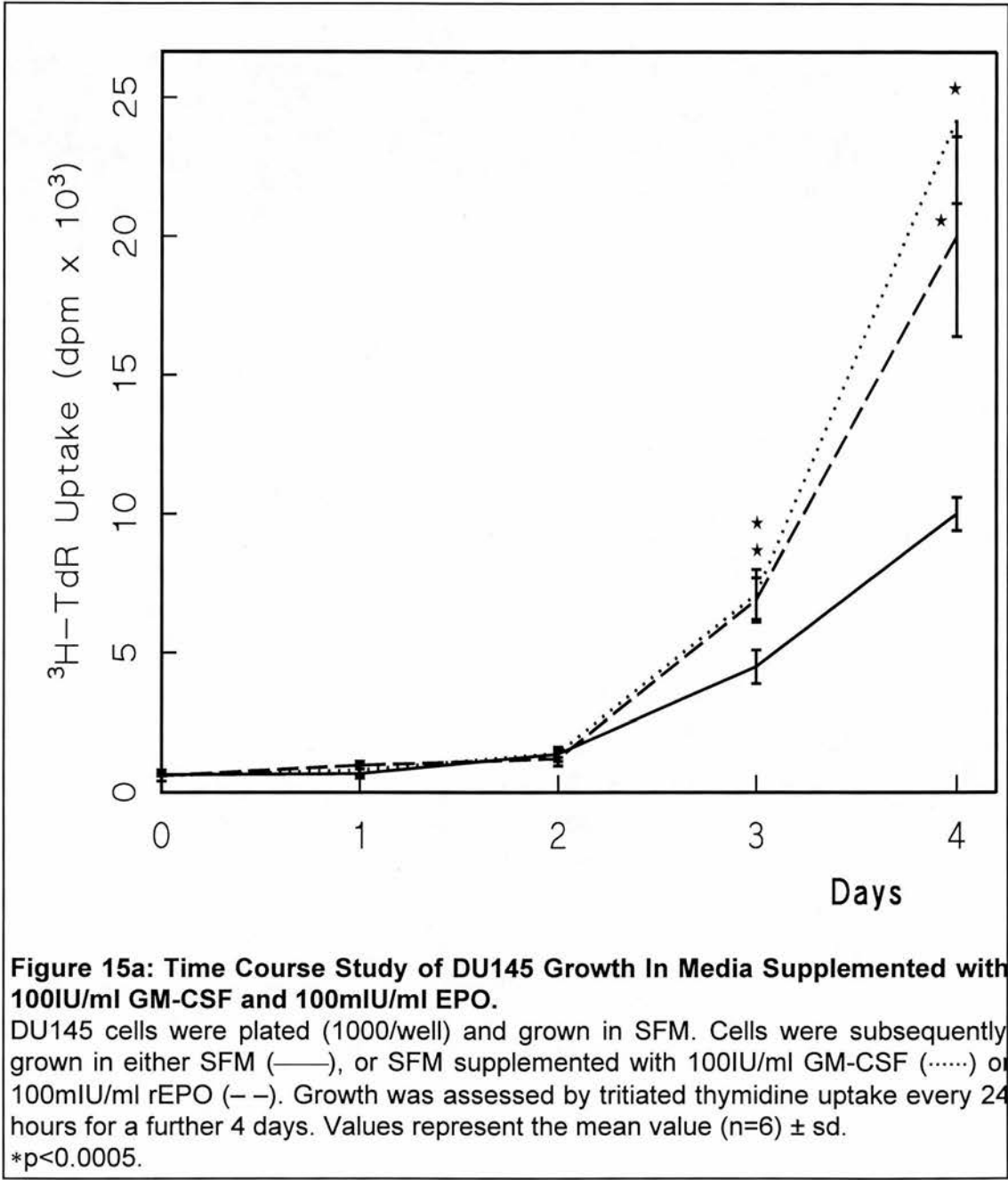
Time course studies were undertaken to gauge the effects of stimulatory concentrations of rEPO (100mIU/ml) and rGM-CSF (100IU/ml) on the growth of DU145 (figure 15a) and PC-3 (figure 15b) cells over a series of days. Growth was measured every 24 hours for 4 days using tritiated thymidine uptake as an index of cell number.

Table 5: Dose Response of Prostate cell lines To Haematopoietic Growth Factors.

Prostate cells were plated and exposed to varying concentrations of HGFs, as described in the legends for figures 14a, 14b and 14c. Values indicate the mean growth stimulation as a multiple of growth in HGF/growth in control. For PC-3 and DU145: sd $\leq \pm 10\%$ of the mean, n=6. For LNCaP: sd $\leq \pm 15\%$ of the mean, n=3.

ND = not done. *p<0.05, **p<0.0025, ***p<0.0005.

rG-CSF (IU/ml)	0	0.01	0.1	1.0	10	100	1000
DU145	1	ND	0.97	0.97	1.2	0.9	0.9
PC-3	1	ND	1.1	1	0.9	1.1	1.2
LNCaP	1	1	1.2	1	1	1.2	ND
rIL-3 (mU/ml)							
DU145	1	ND	1	1.2	0.9	1	1
PC-3	1	ND	1.2	1.3	1.3	1.3	1.3
LNCaP	1	1	1.1	1	1.1	1.1	1.1
rGM-CSF (IU/ml)							
DU145	1	1.4**	1.4**	1.5**	1.6**	2.3***	ND
PC-3	1	1.4*	2.1**	2.8***	2.7***	3***	ND
LNCaP	1	1.5	1.3	1.5	1.7*	2**	ND
rEPO (mIU/ml)							
DU145	1	1.4	1.9**	2**	2.1**	2.2**	ND
PC-3	1	1.6*	1.8**	2.4***	2.5***	2.6***	ND
LNCaP	1	1	1.2	1	1.3	1.3	ND



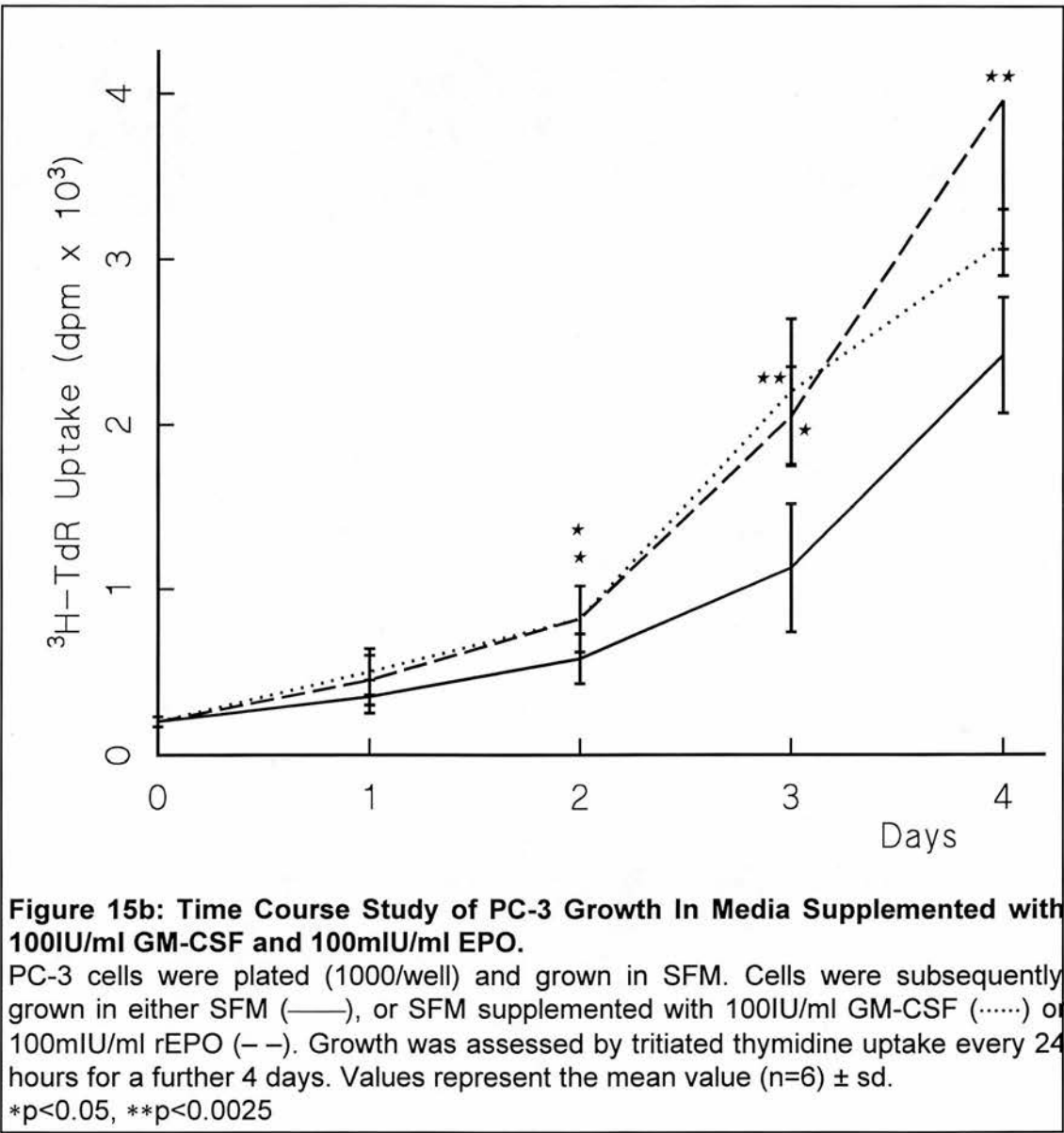


Figure 15a shows that DU145 growth increased over 4 days in all three media tested (i.e., SFM or SFM supplemented with either rGM-CSF or rEPO). Growth in rGM-CSF or rEPO became significantly greater than growth in SFM on days 3 and 4 of culture ($p < 0.0005$). PC-3 cell growth (figure 15b) was stimulated by rEPO and rGM-CSF on all 4 days in culture. The stimulation caused by SFM supplemented with rEPO (100mIU/ml) became significant on day 2 and thereafter (p values indicated on the graph). SFM supplemented with rGM-CSF (100IU/ml) significantly stimulated growth on days 2 and 3. Growth was not assayed further than 4 days in culture because the cells became confluent after this time and were therefore no longer in a log phase of growth.

c) Antibody Inhibition of GM-CSF Stimulated DU145 Growth

To test whether the growth stimulation of prostate cell lines by serum free medium supplemented with 100IU/ml rGM-CSF was specific to rGM-CSF, the HGF was pre-incubated with antibody, before exposing to DU145 cells. The antibody used was a mouse monoclonal raised against human GM-CSF.

Figure 16 illustrates the effect on DU145 growth when the cells were incubated with serum free medium supplemented with increasing concentrations of anti-GM-CSF mAb with or without prior incubation with 100IU/ml rGM-CSF. In a manner similar to previous experiments, when no antibody was present DU145 growth was significantly ($p < 0.025$) stimulated 2.2 fold when exposed to GM-CSF, in comparison to growth in SFM. Increasing concentrations of antibody had no effect on this growth stimulation until 20ng/ml, which caused the stimulation to decrease by 58%.

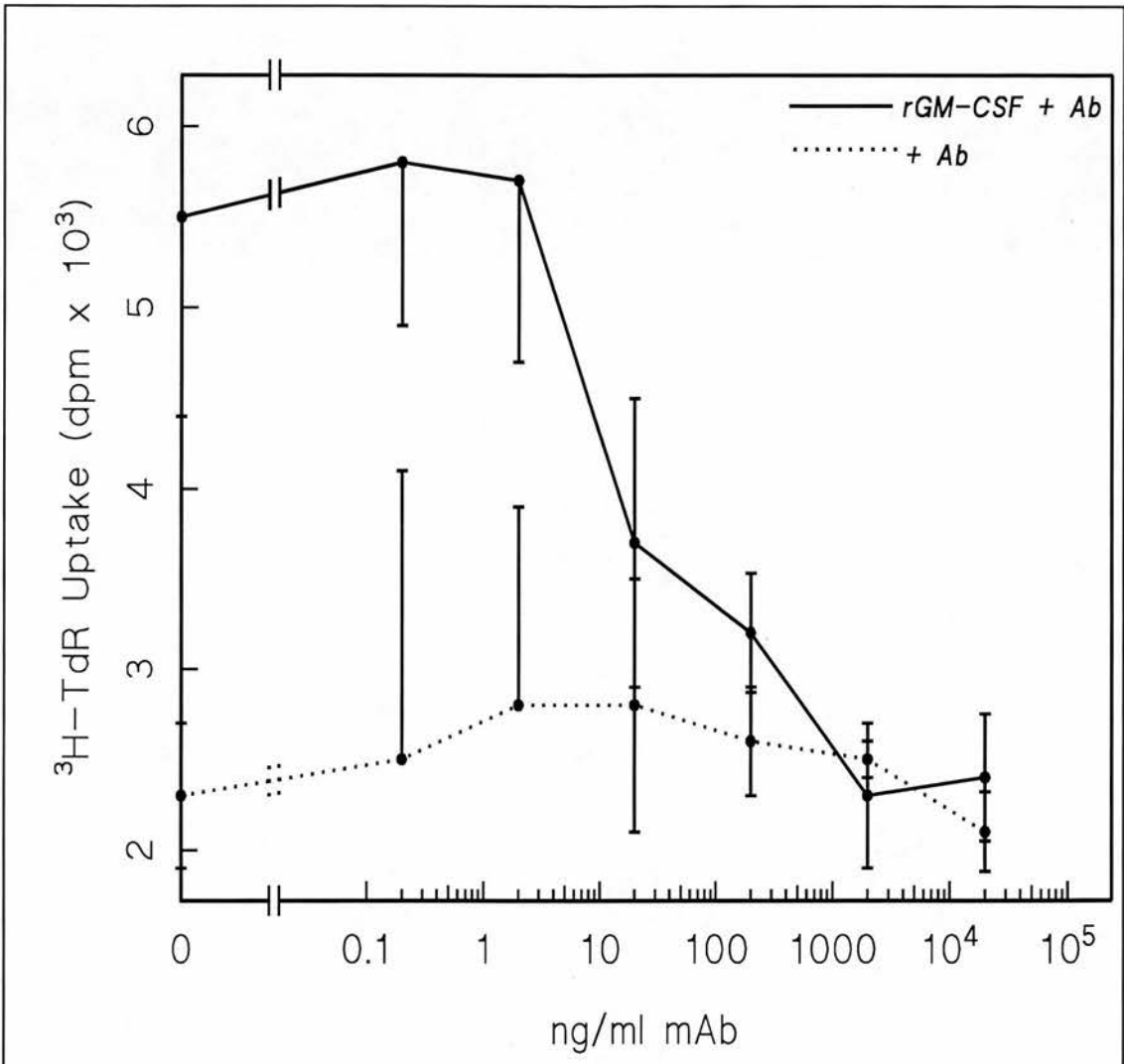


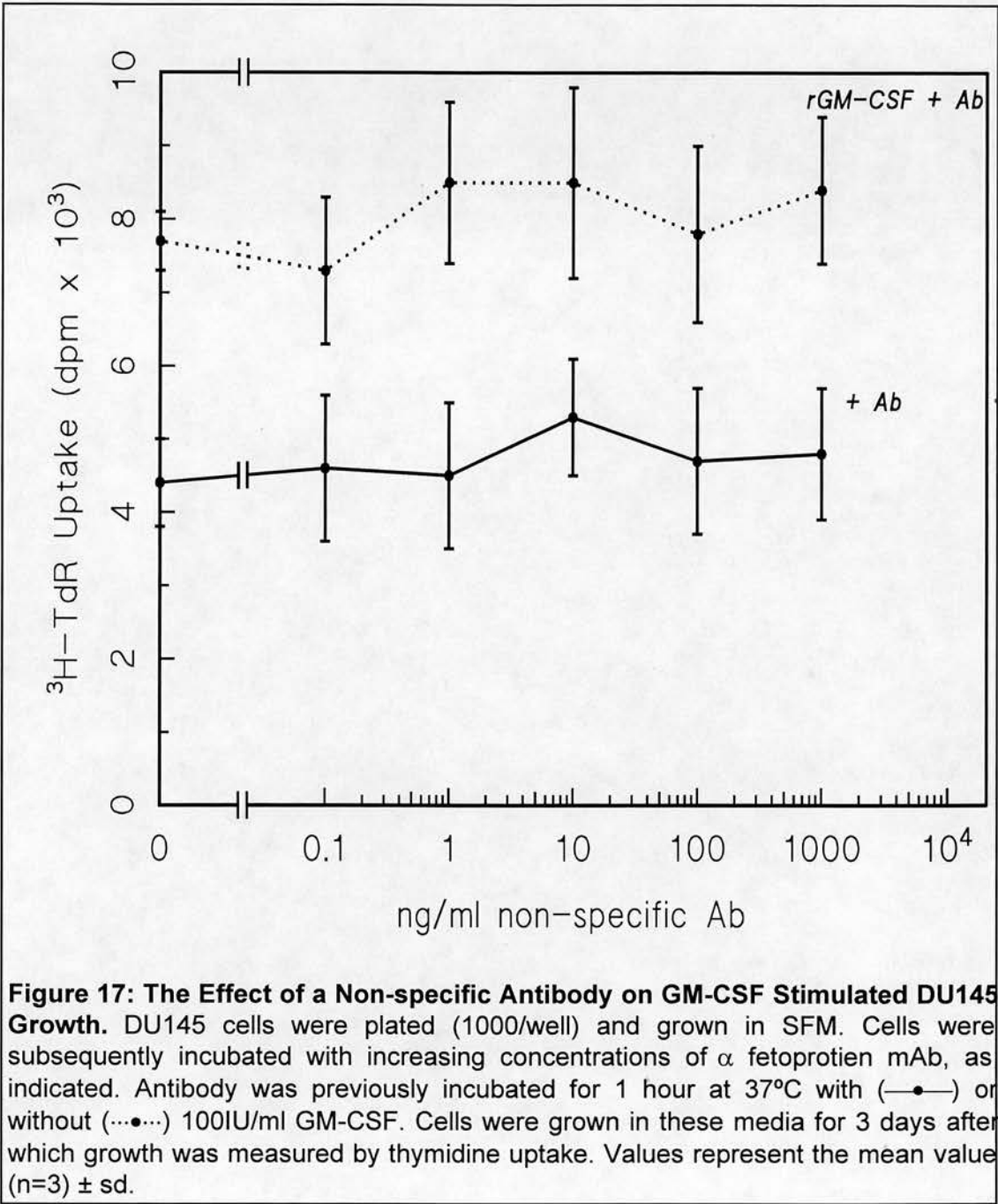
Figure 16: Antibody Inhibition of GM-CSF Stimulated DU145 Growth. DU145 cells were plated (1000/well) and grown in SFM. Cells were subsequently incubated with increasing concentrations of GM-CSF mAb, as indicated. Antibody was previously incubated for 1 hour at 37°C with (—●—) or without (·····) 100IU/ml GM-CSF. Cells were grown in these media for 3 days after which growth was measured by thymidine uptake. Values represent the mean value (n=3) ± sd.

Additional concentrations of antibody caused the growth stimulation to decrease further, and with 2µg/ml of antibody the stimulation of DU145 growth by 100IU/ml rGM-CSF was completely inhibited.

Incubation of DU145 cells in medium supplemented only with increasing concentrations of mAb showed no effect on growth. The highest concentration of mAb tested (20µg/ml) appeared to inhibit basal growth. This inhibition was not significant compared to growth in serum free medium alone, but was significantly reduced ($p < 0.05$) compared to growth in medium supplemented with 2µg/ml of mAb.

In an identical experiment, a non-specific mAb (mouse, IgG₁, raised against α fetoprotein) showed no inhibition of the GM-CSF stimulation (figure 17) emphasising the specificity of GM-CSF to stimulate DU145 growth.

These experiments indicate that serum free medium supplemented with 100IU/ml rGM-CSF could stimulate the growth of DU145 cells and that this could be prevented with 2µg/ml of mAb raised against human GM-CSF. No similar experiment was performed for EPO since an antibody capable of inhibiting biological activity was unavailable at the time these experiments were performed. This also prevented further experiments with EPO, and therefore subsequent experiments concentrated on GM-CSF.

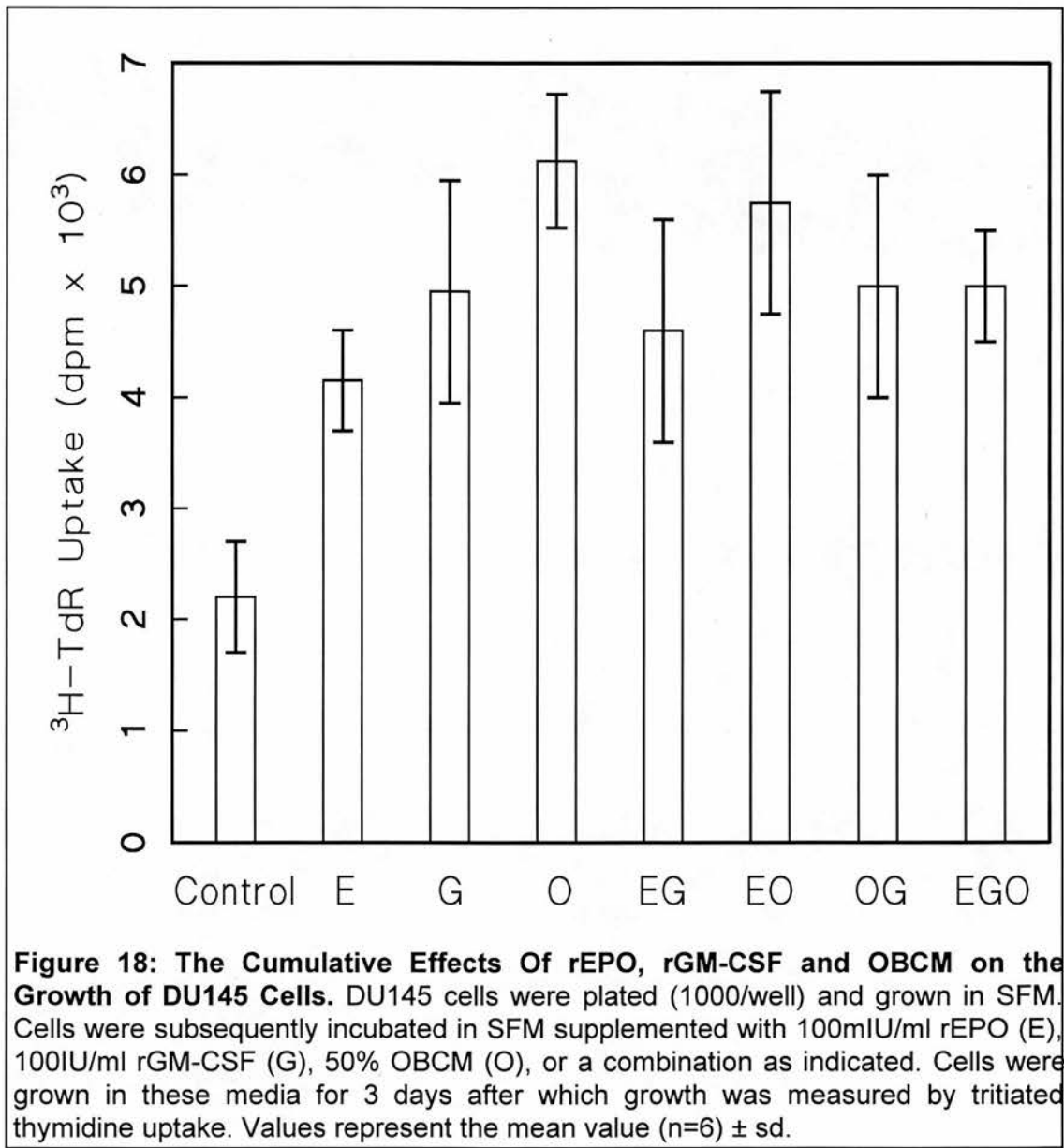


3.4 Is GM-CSF Responsible For The OBCM Growth Stimulation Of Prostate Cell Lines?

Osteoblast-like cell CM, rEPO and rGM-CSF have all been found to stimulate the growth of prostate cell lines. Since GM-CSF is known to be secreted by osteoblast cells (Horowitz *et al* 1989), studies were carried out to establish whether GM-CSF or EPO or other factors could be the stimulatory agents found in OBCM.

The cumulative effects of 50% OBCM, 100IU/ml rGM-CSF and 100IU/ml rEPO on DU145 growth were studied. Typical results are shown in figure 18.

SFM supplemented with 100IU/ml rEPO stimulated growth 1.9 fold compared to control levels of SFM. Medium containing 100IU/ml rGM-CSF stimulated growth 2.2 fold, and 50% OBCM stimulated growth 2.8 fold. All of these were significant ($p < 0.03$). If medium was supplemented with any combination of growth factor or OBCM there were no significant additive effects on the growth stimulation of DU145 cells. Although growth in 100IU/ml of rEPO was significantly less than growth in OBCM, growth in a solution containing both was not significantly different from growth in either solution alone. This suggests that all three agents were stimulating the growth of the DU145 cells to their maximum or that EPO or GM-CSF are already present in the OBCM. To clarify this, the presence of GM-CSF in OBCM was examined by incubating OBCM with the antibody raised against GM-CSF, to see if this affected OBCM induced DU145 growth stimulation.



From previous studies, the stimulation of DU145 cells by 100IU/ml rGM-CSF (equivalent to 10ng/ml rGM-CSF) was inhibited with 2µg/ml of mAb specific to GM-CSF. Other investigators have shown that CM taken from the best natural sources contains 1-10ng/ml GM-CSF (Nicola 1989) therefore 2µg/ml of GM-CSF mAb was thought ample to inhibit any amounts of GM-CSF secreted by the osteoblast-like cell cultures.

Subsequently DU145 cells were incubated with increasing concentrations of OBCM (diluted in SFM) with or without pre-incubation with 2µg/ml of anti-GM-CSF mAb. These results are shown in figure 19 and indicate that there was no significant difference between the growth of DU145 cells in OBCM with or without pre-incubation with anti-GM-CSF mAb.

Therefore the mAb demonstrated no significant inhibition of the stimulatory action of OBCM. This result was similar when repeated with CM from a different osteoblast-like cell culture.

The stimulatory fractions of OBCM (>100Kd and <100Kd), found in the previous section (3.2 d), were also pre-incubated with 2µg/ml of anti-GM-CSF mAb, before exposing them to DU145 cells (figure 20). The results indicated that the fractions of OBCM >100Kd and <100Kd significantly stimulated DU145 growth 2.3 fold and 1.4 fold, respectively (p values are indicated on the graph). However, these growth stimulations were not inhibited by pre-incubating the fractions with monoclonal antibody specific for human GM-CSF.

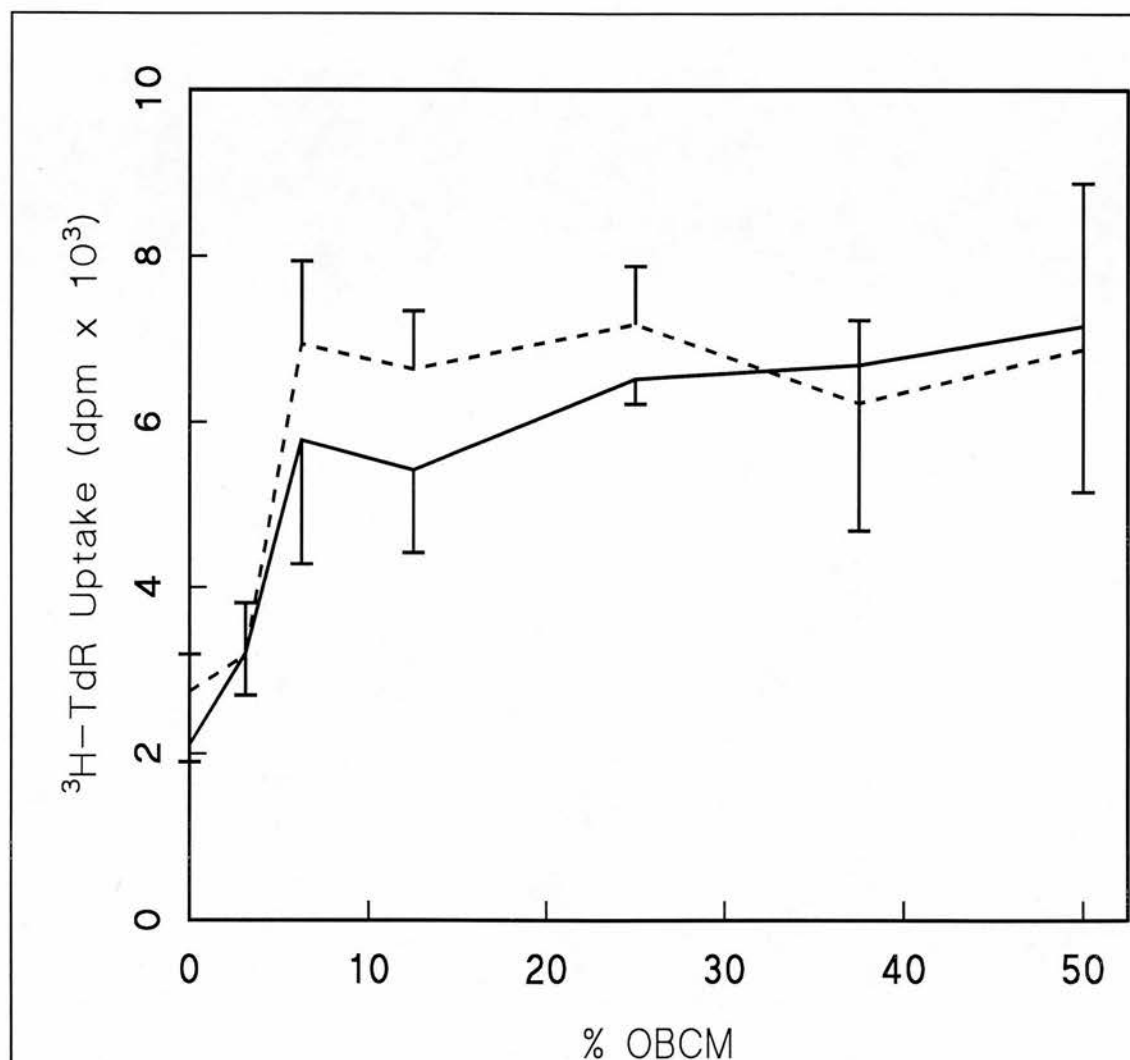
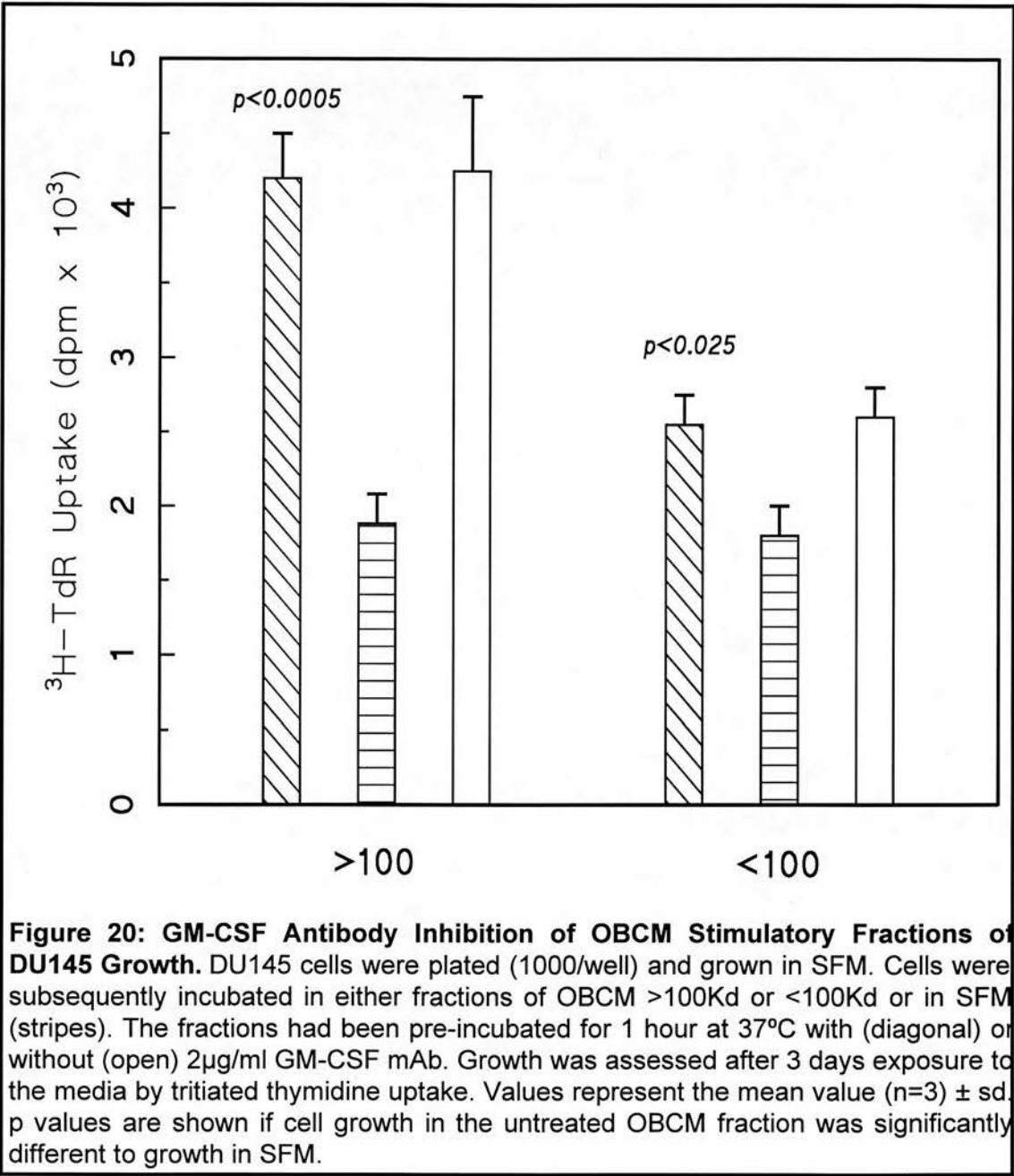


Figure 19: GM-CSF Antibody Inhibition OBCM Stimulated DU145 Growth.

DU145 cells were plated (1000/well) and grown in SFM. Cells were subsequently incubated in increasing concentrations of OBCM (diluted with SFM), as indicated, which were pre-incubated for 1 hour at 37°C with (- - -) or without (—) 2 µg/ml GM-CSF mAb. Cells were grown in these media for 3 days, growth was then measured by tritiated thymidine uptake. Values represent the mean value (n=3) ± sd.



3.5 Secretion Of GM-CSF by Prostate Cell Lines

Various investigators have reported that GM-CSF can be secreted by non-haematopoietic cells and may have a role in cancer progression (Takeda *et al* 1991, Young *et al* 1992, Pekarek *et al* 1993). Therefore to investigate further the involvement of GM-CSF in prostate cancer the next experiments examined whether GM-CSF was produced from the prostate carcinoma cell lines.

To determine if prostate cell lines can produce GM-CSF, the presence of protein was analysed by immunohistochemistry, ELISA and Western Blotting. GM-CSF gene transcripts were also investigated by RT PCR of total RNA extracted from the prostate cell lines.

a) Immunohistochemical staining of cell lines with a mouse monoclonal GM-CSF antibody

Cells which were ~ 50% confluent were immunohistochemically stained to determine the presence of GM-CSF. Staining was kindly analysed by Dr. David Hughes from the Pathology Dept., University of Edinburgh. The following plates illustrate the pattern of DAB-peroxidase staining due to the binding of a mouse mAb raised to human GM-CSF, after incubation with a variety of cell lines:

Plate 2: Illustrates a negative control for DU145 cells, this was the entire immunostain procedure without primary antibody. No positive staining was evident, only the blue nuclear stain of haematoxylin. All other cells were similarly stained so that comparisons of positive stains could be made, however these were not included in the thesis since they all indicated a negative result, identical to this plate.

Plate 3: Indicates positive staining of DU145 cells, produced when primary antibody was included in the staining procedure. Positive staining was seen throughout the cytoplasm.

Plate 4: PC-3 cells also showed positive staining which was more specific to the perinuclear region. This type of staining is indicative of a secretory protein.

Plate 5: The LNCaP cell line demonstrated no apparent positive staining, above background levels.

Plate 6: Osteoblast-like cells were used as positive controls (they are known to secrete GM-CSF [Horowitz *et al* 1989]). These cells showed positive staining throughout the cytoplasm, which became stronger in the perinuclear region.

Plate 7: The osteosarcoma cell line, MG63, was also used as a positive control. This plate illustrates that a weak positive cytoplasmic and perinuclear stain was produced.



Plate 2: Blank Immunostain of DU145 Cells. 50% confluent DU145 cells underwent immunostaining with no primary antibody and were counter stained with Mayers haematoxylin. x10 magnification.

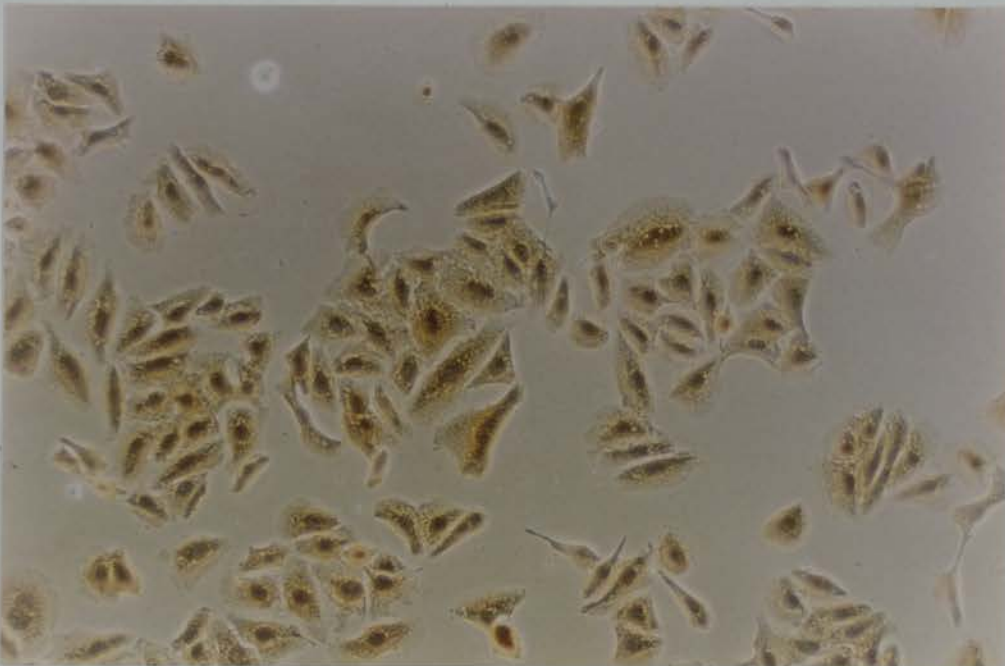


Plate 3: GM-CSF Immunostaining of DU145 Cells. 50% confluent DU145 cells were immunostained with GM-CSF mAb (1:200), and counter stained with Mayers haematoxylin. x10 magnification.

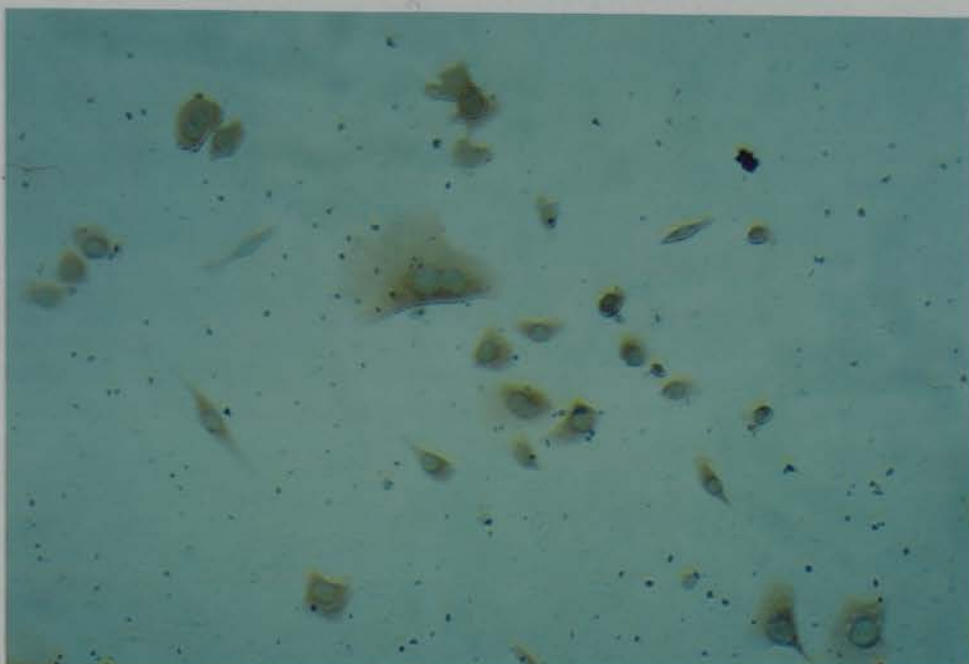


Plate 4: GM-CSF Immunostaining of PC-3 Cells. 50% confluent PC-3 cells were immunostained with GM-CSF mAb (1:200), and counter stained with Mayer's haematoxylin. x10 magnification.

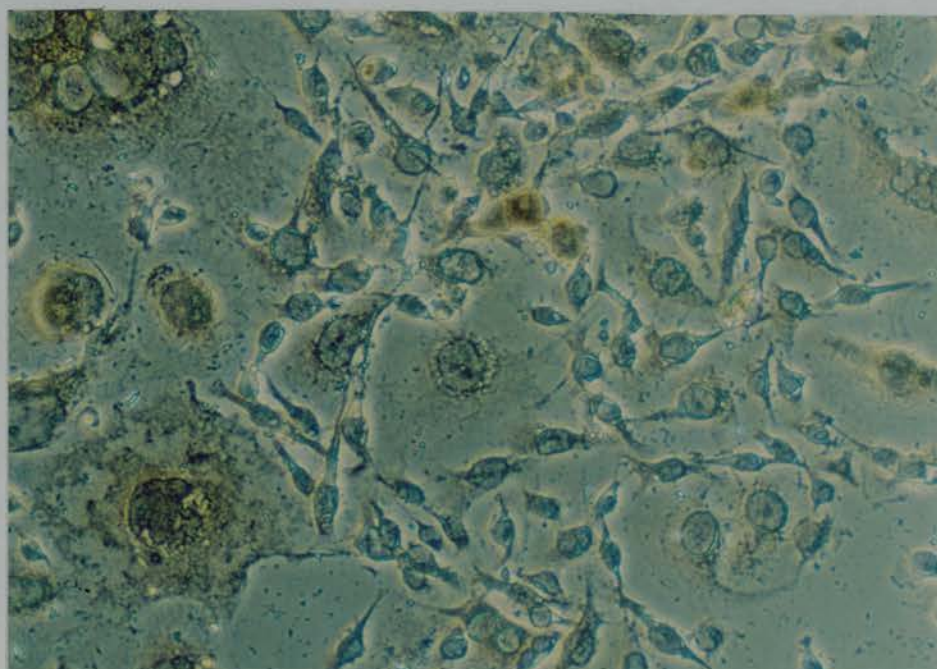


Plate 5: GM-CSF Immunostaining of LNCaP Cells. 50% confluent LNCaP cells were immunostained with GM-CSF mAb (1:200), and counter stained with Mayer's haematoxylin. x10 magnification.



Plate 6: GM-CSF Immunostaining of Osteoblast-like Cells. 50% confluent osteoblast-like cells were immunostained with GM-CSF mAb (1:200), and counter stained with Mayers haematoxylin. x10 magnification.



Plate 7: GM-CSF Immunostaining of MG63 Cells. 50% confluent MG63 cells were immunostained with GM-CSF mAb (1:200), and counter stained with Mayers haematoxylin. x10 magnification.

A variety of cell lines derived from cancers other than prostate were also tested for the presence of GM-CSF by immunostaining. The results are summarised in table 6, an example of positive (HF19) and negative (CHANG) staining are shown in plates 8 and 9 respectively.

Table 6: Immunohistochemical staining of cell lines with a mouse monoclonal GM-CSF antibody

Cell Line	Staining	Plate
WISH (melanoma)	Positive	9
CHANG (liver)	Negative	
RT112 (bladder)	Negative	
HEP2 (larynx)	Negative	8
HF19 (lung)	Positive	
HOS (osteosarcoma)	Negative	

i) Analysis of the Specificity of GM-CSF Staining

To check the specificity of staining produced by the anti-GM-CSF mAb, the mAb was pre-incubated with excess GM-CSF. The lowest titration of antibody to produce a positive stain (1:800) was used in order that excess concentrations of rGM-CSF could be added.

Results are shown for staining DU145 cells with; no antibody (plate 10), GM-CSF mAb (plate 11), GM-CSF mAb pre-incubated with excess GM-CSF (plate 12), GM-CSF mAb pre-incubated with excess IL-3 (plate 13), GM-CSF mAb pre-incubated with excess EPO (plate 14).

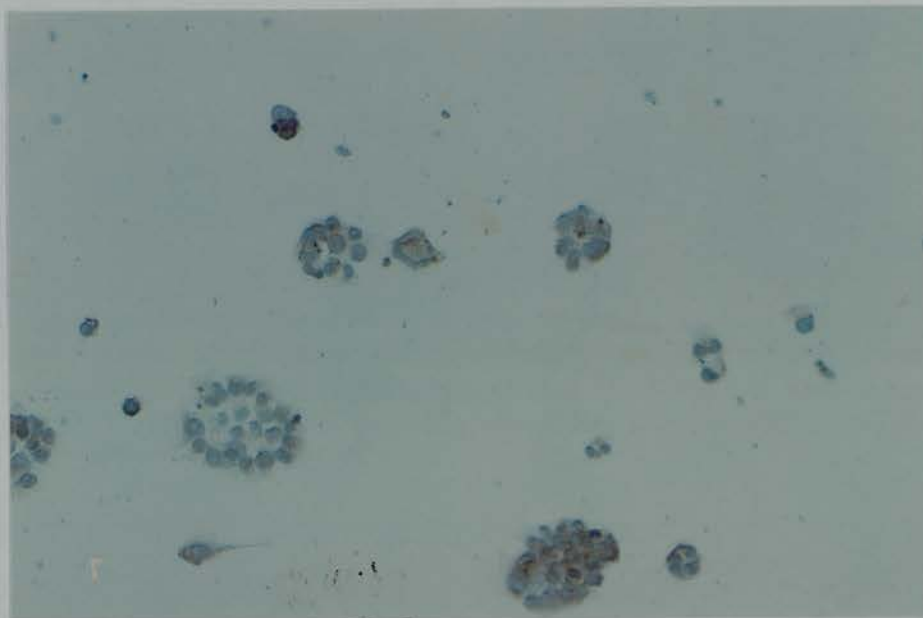


Plate 8: GM-CSF Immunostaining of HF19 Cells. 50% confluent HF19 cells were immunostained with GM-CSF mAb (1:200), and counter stained with Mayer's haematoxylin. x10 magnification.

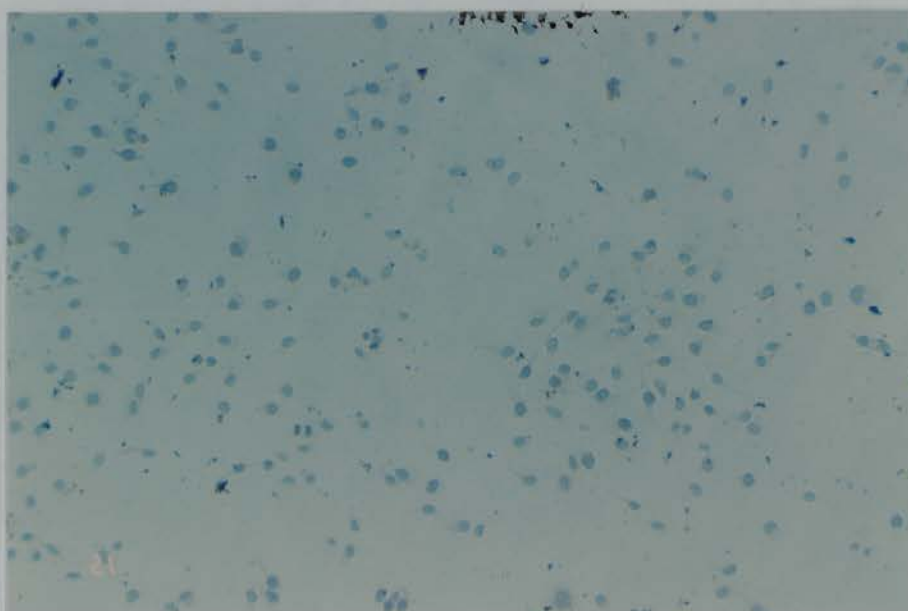


Plate 9: GM-CSF Immunostaining of CHANG Cells. 50% confluent CHANG cells were immunostained with GM-CSF mAb (1:200), and counter stained with Mayer's haematoxylin. x10 magnification.



Plate 10: Blank Immunostain of DU145 Cells. 50% confluent DU145 cells underwent immunostaining with no primary antibody and were counter stained with Mayer's haematoxylin. x10 magnification.

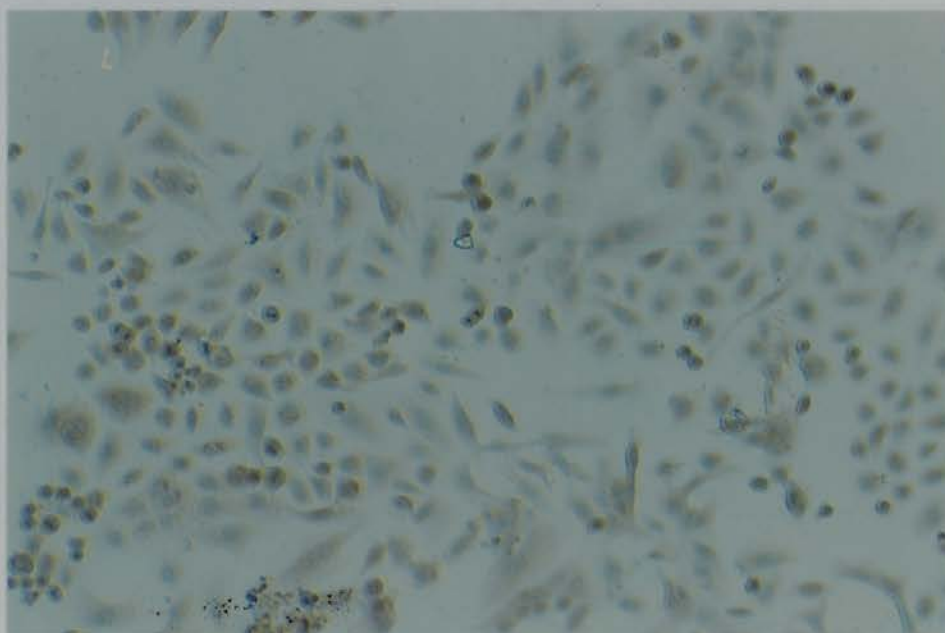


Plate 11: GM-CSF Immunostaining of DU145 Cells. 50% confluent DU145 cells were immunostained with GM-CSF mAb (1:800) and counter stained with Mayer's haematoxylin. x10 magnification.



Plate 12: GM-CSF mAb Incubated with excess GM-CSF Immunostaining of DU145 Cells. 50% confluent DU145 cells were immunostained with GM-CSF mAb (1:800), previously incubated with excess rGM-CSF and counter stained with Mayer's haematoxylin. x10 magnification.

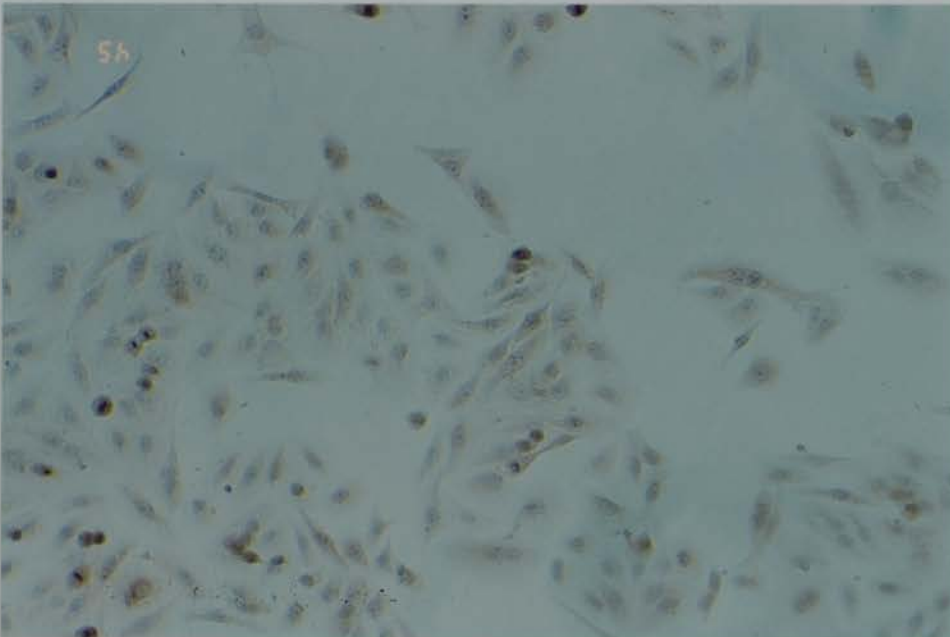


Plate 13: GM-CSF mAb Incubated with excess IL-3 Immunostaining of DU145 Cells. 50% confluent DU145 cells were immunostained with GM-CSF mAb (1:800), previously incubated with excess rIL-3 and counter stained with Mayer's haematoxylin. x10 magnification.



Plate 14: GM-CSF mAb Incubated with excess EPO Immunostaining of DU145 Cells. 50% confluent DU145 cells were immunostained with GM-CSF mAb (1:800), previously incubated with excess rEPO and counter stained with Mayers haematoxylin. x10 magnification.

The results indicate that the positive staining of DU145 cells by GM-CSF mAb can only be inhibited by prior incubation of the mAb with excess GM-CSF. Pre-incubation with either EPO or IL-3 did not lead to inhibition of the stain, indicating the staining is very specific to GM-CSF.

ii) Immunohistochemical GM-CSF Staining of Prostate Cancer Primary Cultures and Tissue Sections

The positive staining of prostate cell lines (PC-3 and DU145) led to further immunohistochemical investigations of prostate carcinoma primary cultures and also prostate tissue sections.

Primary cultures of epithelia and fibroblasts were produced from prostate chips which had been identified as carcinoma, from pathology reports. These were stained with mAb specific to GM-CSF, in an identical manner to the cell lines. The results were as follows:

Plate 15: Epithelial cultures of prostate cancer showed positive staining throughout the cytoplasm, which became stronger in perinuclear and nuclear regions.

Plate 16: Fibroblast cultures from the same prostate cancer chips showed no evidence of staining.

These results were reproduced on one other occasion using 'chips' of prostate carcinoma from a different patient.



Plate 15: GM-CSF mAb Immunostaining of Primary Cultures of Prostate Carcinoma Epithelia. 50% confluent prostate carcinoma epithelia cells were immunostained with GM-CSF mAb (1:200) and counter stained with Mayer's haematoxylin. x10 magnification.

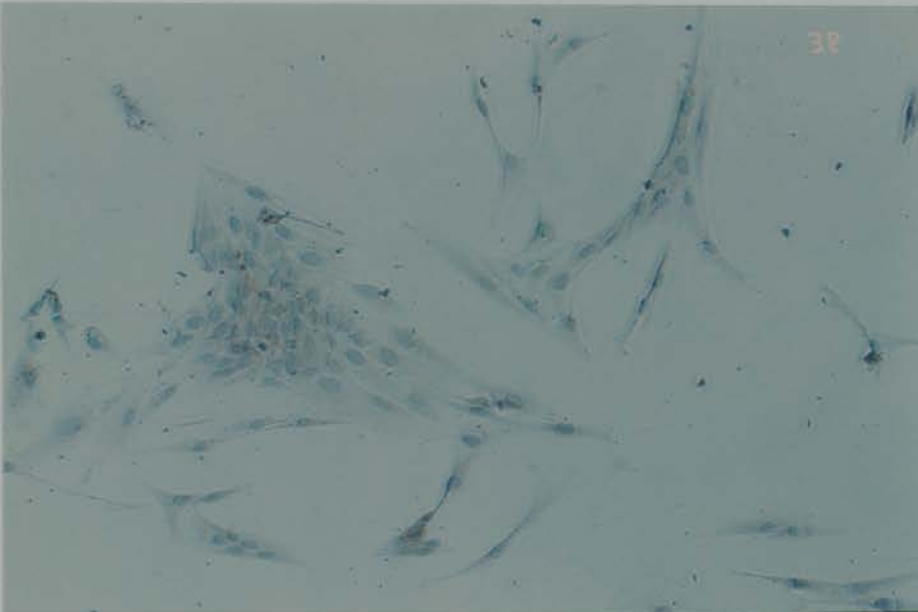


Plate 16: GM-CSF mAb Immunostaining of Primary Cultures of Prostate Carcinoma Fibroblasts. 50% confluent prostate carcinoma fibroblast cells were immunostained with GM-CSF mAb (1:200) and counter stained with Mayer's haematoxylin. x10 magnification.

Paraffin sections of prostate and tonsil tissue were also immunostained with GM-CSF mAb:

Plate 17: This indicates the staining pattern of normal peripheral zone prostate (34-40 year old). This area of the prostate was chosen since it is the commonest site of carcinoma origin (McNeal *et al* 1988b). It exhibited staining specific to the basement membrane (BM).

Plate 18: Benign prostatic hyperplasia sections indicated no specific staining, though the double epithelial layer of the prostate ducts was clearly seen.

Plate 19: Well differentiated carcinoma of the prostate (Gleason score 3) exhibited no specific staining.

Plate 20: Poorly differentiated prostate carcinoma (Gleason score 9) showed strong positive staining amongst the malignant epithelia, whereas the stromal areas remained negative. Haematopoietic cells, visible in small blood vessels, also stain strongly, providing an internal control. Areas of smooth muscle also stained positively in both poor and well differentiated carcinoma sections.

Plate 21: Tonsil lymph nodes were stained since they contain activated T-lymphocytes and therefore, provided a positive control. Strong positive staining was found mainly in the paracortex (the expected site of activated T-lymphocytes) though weaker staining was also seen in the endothelia.

These results have each been reproduced on three separate sets of each tissue type.

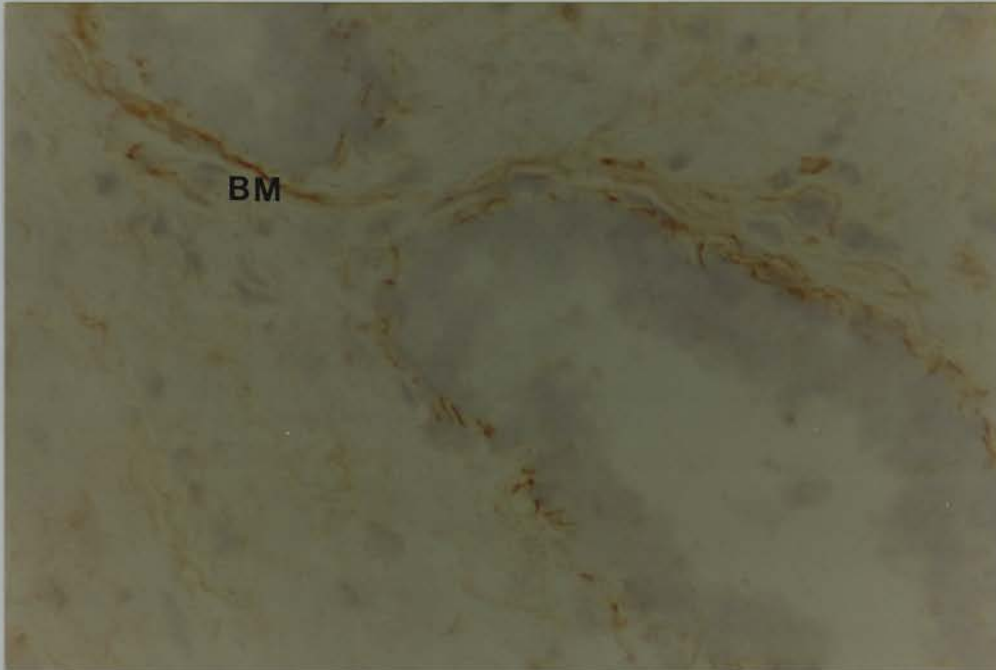


Plate 17: GM-CSF mAb Immunostaining of Normal Peripheral Zone Prostate Gland. Tissue sections of normal peripheral zone prostate were fixed in acetone, immunostained with GM-CSF mAb (1:400) and counter stained with Mayers haematoxylin. x40 magnification.

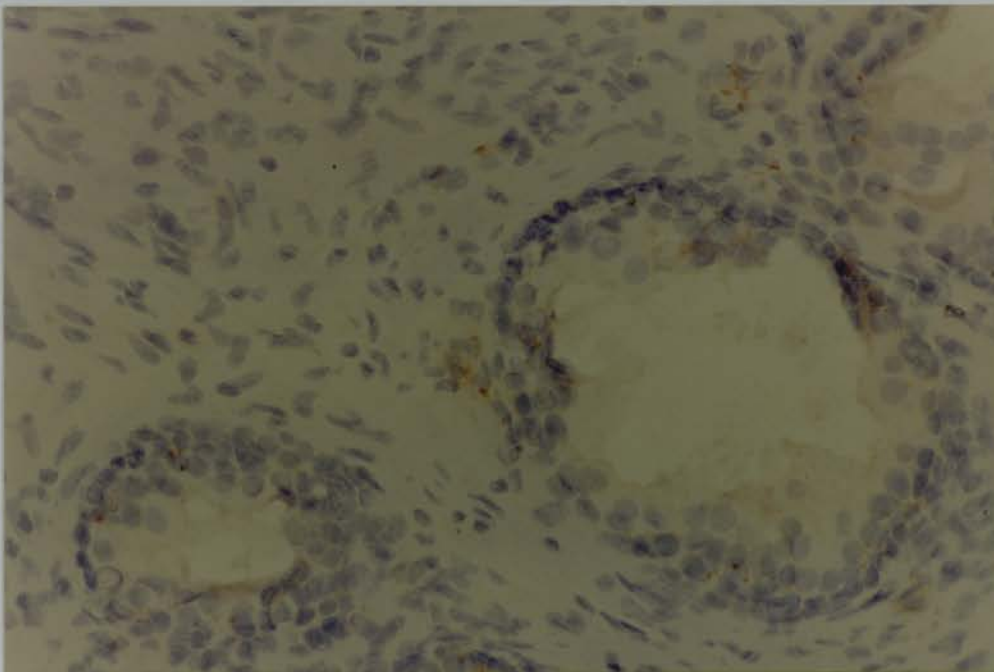


Plate 18: GM-CSF mAb Immunostaining of Benign Prostatic Hyperplasia Tissue Sections. Paraffin sections of benign prostatic hyperplasia were immunostained with GM-CSF mAb (1:400) and counter stained with Mayers haematoxylin. x40 magnification.

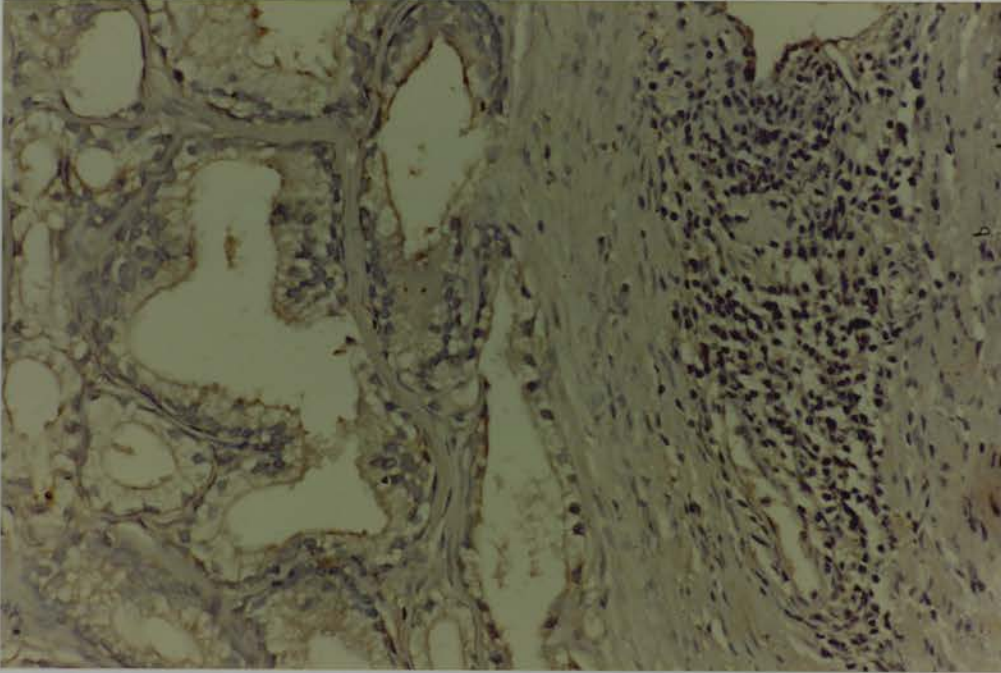


Plate 19: GM-CSF mAb Immunostaining Well Differentiated Prostate Carcinoma Tissue Sections. Paraffin sections of well differentiated prostate carcinoma were immunostained with GM-CSF mAb (1:400) and counter stained with Mayer's haematoxylin. x25 magnification.

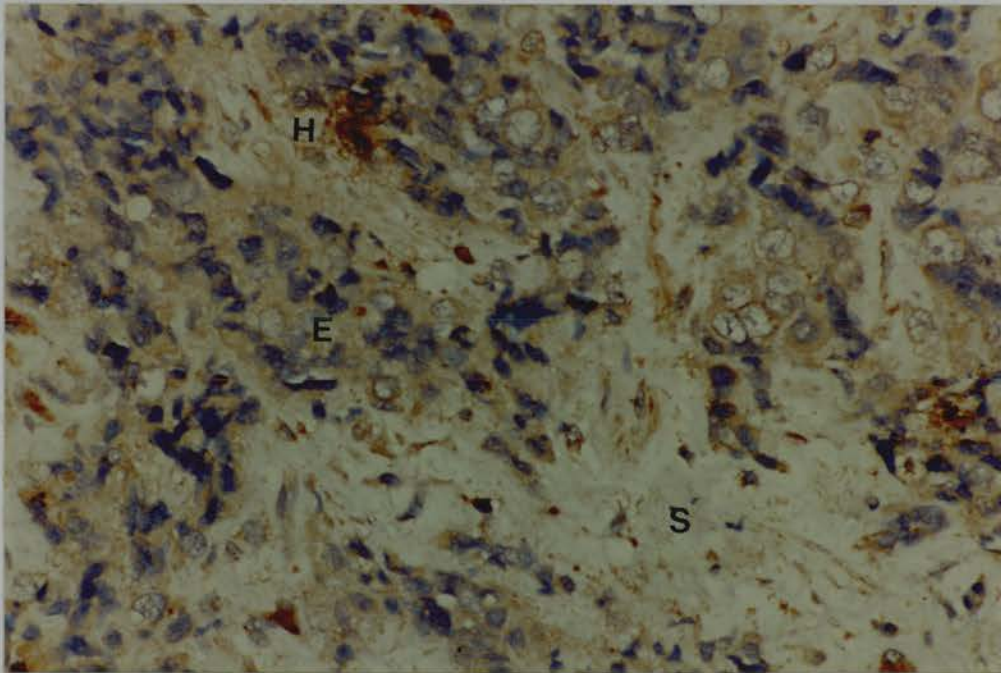


Plate 20: GM-CSF mAb Immunostaining of Poorly Differentiated Prostate Carcinoma tissue Sections. Paraffin sections of poorly differentiated prostate carcinoma were immunostained with GM-CSF mAb (1:400) and counter stained with Mayer's haematoxylin. x40 magnification. E, epithelial; S, stroma; H, haematopoietic cell.

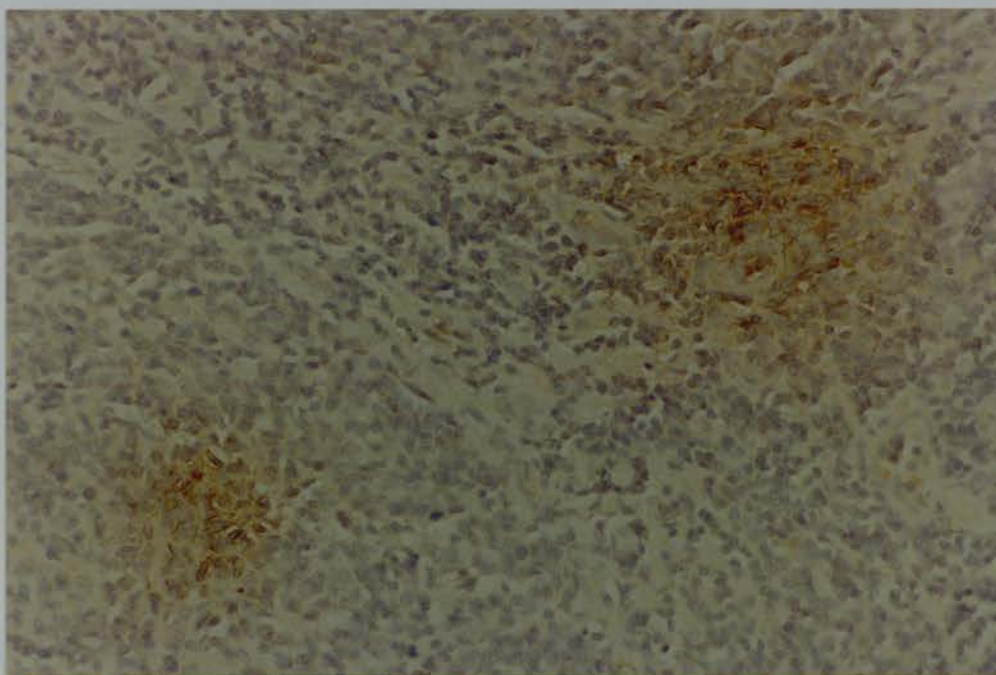


Plate 21: GM-CSF mAb Immunostaining of Tonsil Tissue Sections. Paraffin sections of human tonsil carcinoma were immunostained with GM-CSF mAb (1:400) and counter stained with Mayer's haematoxylin. x25 magnification.

b) GM-CSF Immunoassay of Prostate Cell Line Conditioned Medium

To assess whether or not the GM-CSF-like material detected in PC-3 and DU145 cultures was produced by these cells or not, media conditioned by the cells was collected and analysed by ELISA. Medium conditioned by confluent cultures of LNCaP, MG63, benign prostatic fibroblasts and osteoblast-like cells was also investigated. The amount of GM-CSF-like material in all CM was assayed using a commercial ELISA kit. The kit antibody exhibited no cross-reactivity with TGF α , TGF β 1, EPO, IL-3 or G-CSF, at the concentrations indicated (table 7).

The results summarised in table 8 are typical of those produced in three separate experiments, each time using a fresh sample of CM.

GM-CSF-like material was detected in pg quantities/ μ g protein of media conditioned by PC-3 or DU145 cells. If either CM was diluted, the levels of GM-CSF-like material detected were diluted in parallel. There were no detectable levels of GM-CSF in LNCaP CM or benign fibroblast CM, whilst it was found in media conditioned by the positive controls, MG63 and osteoblast-like cells. No detectable levels of GM-CSF were found in serum free medium.

c) Expression of GM-CSF-like Material By Western Blotting

The results so far have suggested that the prostate cell lines DU145 and PC-3 can produce GM-CSF-like material, this was further established by analysing media conditioned by DU145 and PC-3 cell lines with Western blot. Proteins in these conditioned media were size fractionated on a reducing polyacrylamide gel, and, after blotting, GM-CSF protein bands were specifically probed with a mAb.

Table 7: Results indicating lack of ELISA antibody cross-reactivity.

The indicated concentrations of growth factors were tested with the ELISA kit for the presence of GM-CSF.

Growth Factor	Protein ($\mu\text{g/ml}$)	GM-CSF (pg/ml)	GM-CSF ($\text{pg}/\mu\text{g protein}$)
TGF α	0.5	0	0
TGF $\beta 1$	0.0005	0	0
EPO	0.08	0	0
IL-3	0.01	0	0
G-CSF	0.1	0	0

Table 8 : GM-CSF Immunoassay Of Prostate Cancer Cell Line Conditioned Medium.

Cell lines and primary cultures were incubated in serum free medium for 24 hours. The conditioned medium was collected, filtered and tested for the presence of GM-CSF using a commercial ELISA kit. Protein concentration of the CM was assayed using the Bradford method. Values represent the mean \pm standard deviation, $n=3$ (for protein concentrations sd were $\leq \pm 10\%$ of the mean).

Conditioned media	GM-CSF (pg/ml)	Protein ($\mu\text{g/ml}$)	GM-CSF ($\text{pg}/\mu\text{g protein}$)
DU145	214 ± 1.6	130	1.7
" " diluted x2	110 ± 0.7	60	1.8
PC-3	535 ± 4.7	210	2.5
" " diluted x2	282 ± 2.5	107	2.6
LNCaP	0	140	0
BPH Fibroblasts	0	154	0
MG63	170 ± 2.2	108	1.6
Osteoblast-like cell	21 ± 0.4	150	0.14

The results are shown in plate 22. Protein bands (immunoreactive with the anti-GM-CSF mAb) were evident from media conditioned by PC-3 (B), DU145 (D), MG63 (F) and osteoblast-like cells (G). All these bands co-migrated with a sample of recombinant GM-CSF (A), at 42.5Kd. No bands were apparent from the LNCaP (C) cell line or benign prostate fibroblasts (E).

Repetition of the immunoblot with a different antibody (polyclonal sheep-anti human GM-CSF) yielded the same results (plate 23). rGM-CSF (lane A) again migrated at 42.5Kd together with protein bands from DU145 CM (B) and PC-3 CM (C). The second immunoblot reinforces the suggestion that the bands are due to GM-CSF.

These bands were consistently produced with different batches of conditioned media and with different dilutions of CM. In further support of these results, a sample of rGM-CSF reconstituted in phosphate buffer plus 0.1% BSA also ran at 42.5Kd. The molecular weight of GM-CSF is 18-22Kd (Wong *et al* 1985) therefore the peptide was apparently running as a dimer. Since the electrophoresis was carried out under reducing conditions and the samples boiled up to 15 minutes it is unlikely a dimer had formed, though this cannot be ruled out. To check the protein was correctly reduced a loading buffer was prepared as follows: 50mM Tris.Cl pH6.8, 100mM dithiothreitol, 10% SDS, 0.1% bromophenol blue, 30% glycerol. Using different concentrations of dithiothreitol (100, 200mM) and SDS (5 and 10%) the band still did not run at 18-22Kd. Considering the sample of rGM-CSF was originally reconstituted with 0.1% BSA, an adduct may have formed.

Plate 24 shows a polyacrylamide gel which was silver stained to show the multitude of proteins present in all the conditioned media.

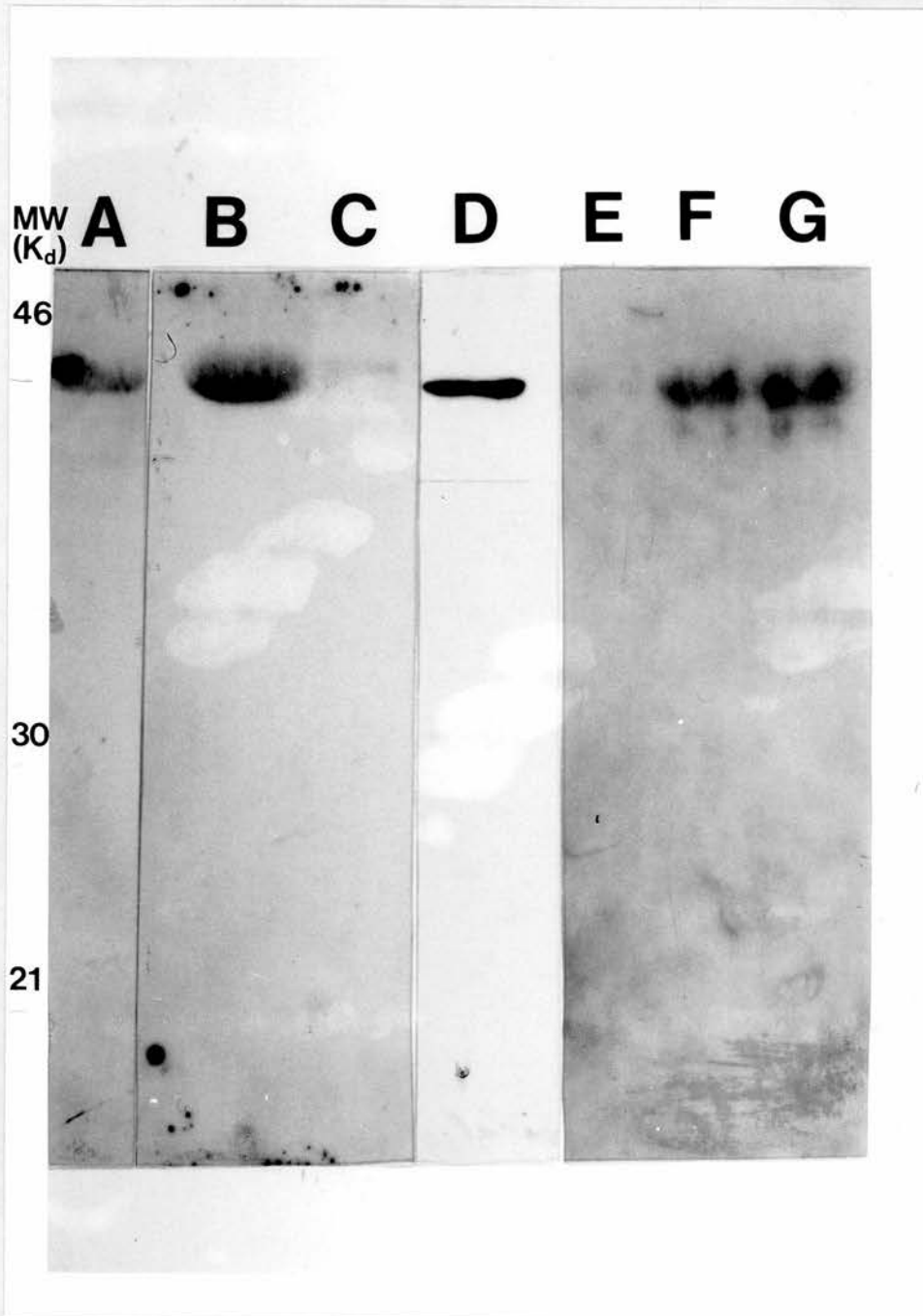


Plate 22: Expression of GM-CSF in Prostate Cell Line CM Analysed by Western Blot. Samples of media conditioned by cell lines were run on a 12% PAGE at 20mA and then immunoblotted with a GM-CSF monoclonal antibody. The lanes are: A, rGM-CSF (25ng); B, PC-3 (10 μ g protein); C, LNCaP (50 μ g protein); D, DU145 (50 μ g protein); E, benign prostate fibroblasts (17 μ g protein); F, MG63 (17 μ g protein); G, osteoblast-like cell (10 μ g protein). MW markers are indicated in Kd.

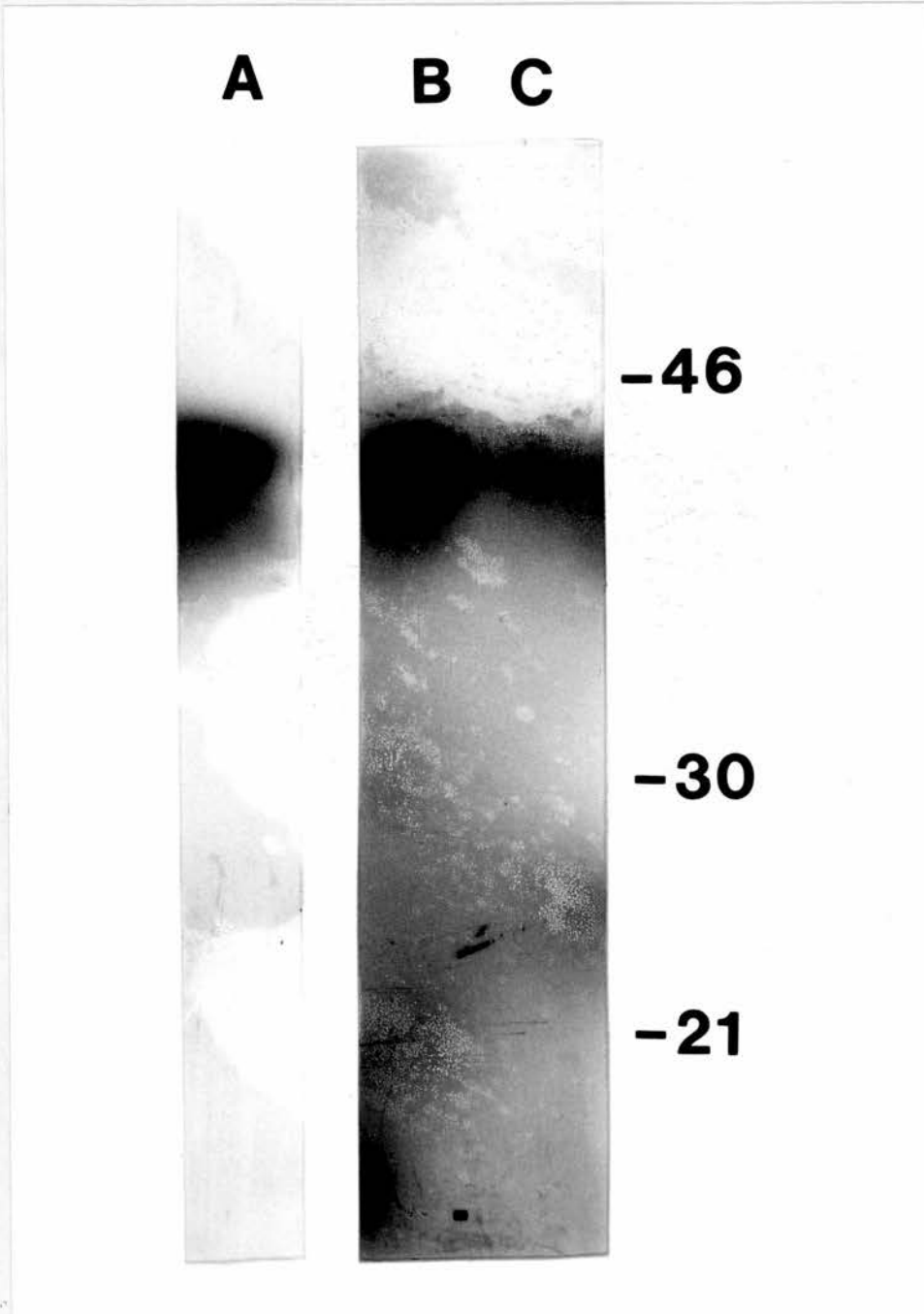


Plate 23: Repetition of Western Blot With A Second GM-CSF Antibody.
Samples of media conditioned by cell lines were run on a 12% PAGE at 20mA and then immunoblotted with a GM-CSF polyclonal antibody.
The lanes are: A, rGM-CSF (25ng); B, DU145 (1 μ g protein); C, PC-3 (5 μ g protein).
MW markers are indicated in Kd.

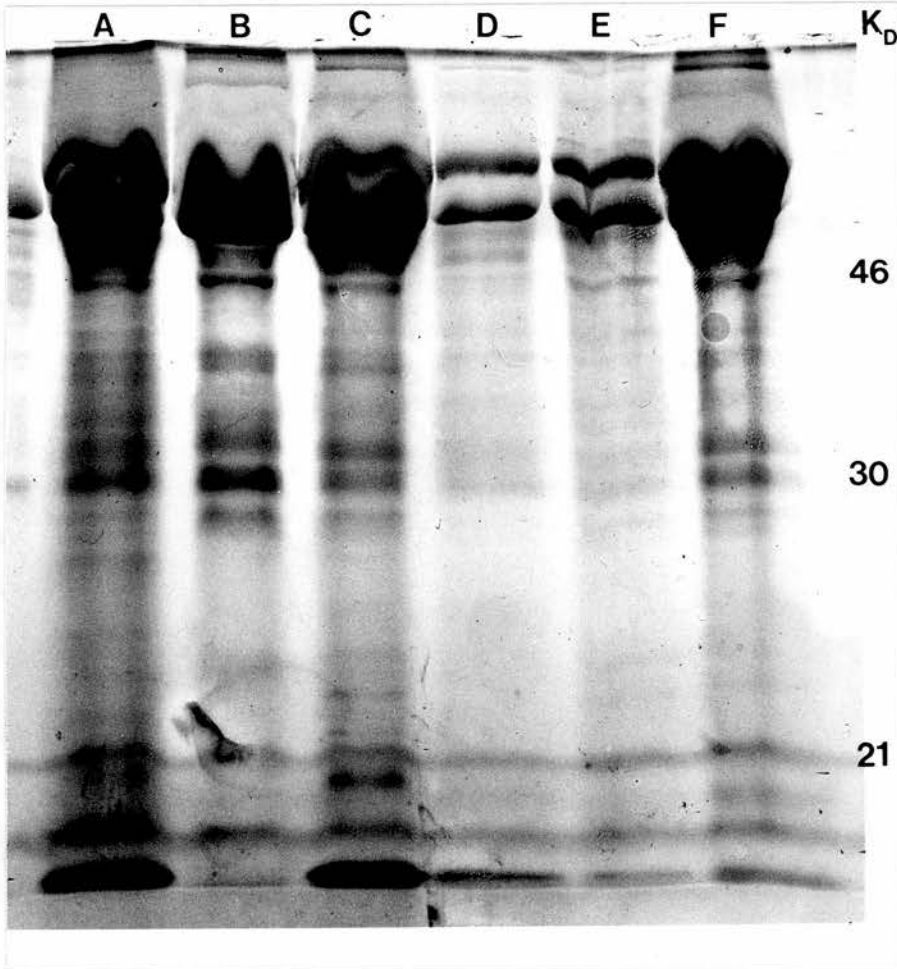


Plate 24: Silver Stain Of Conditioned Media Separated On 12% Polyacrylamide Gel. Samples of conditioned media were run on 12% PAGE and then silver stained to develop the protein bands. The lanes are: A, DU145 (50µg protein); B, PC-3 (10µg protein); C, benign prostatic fibroblasts (17µg protein); D, MG63 (17µg protein); E, osteoblast-like cell (10µg protein); F, LNCaP (17µg protein). MW markers are indicated in Kd.

d) PCR Amplification of GM-CSF Gene Transcripts In Prostate Cell Lines

The production of GM-CSF by DU145 and PC-3 cells but not by LNCaP was explored further by amplifying possible GM-CSF gene transcripts in the three prostate cell lines with RT PCR.

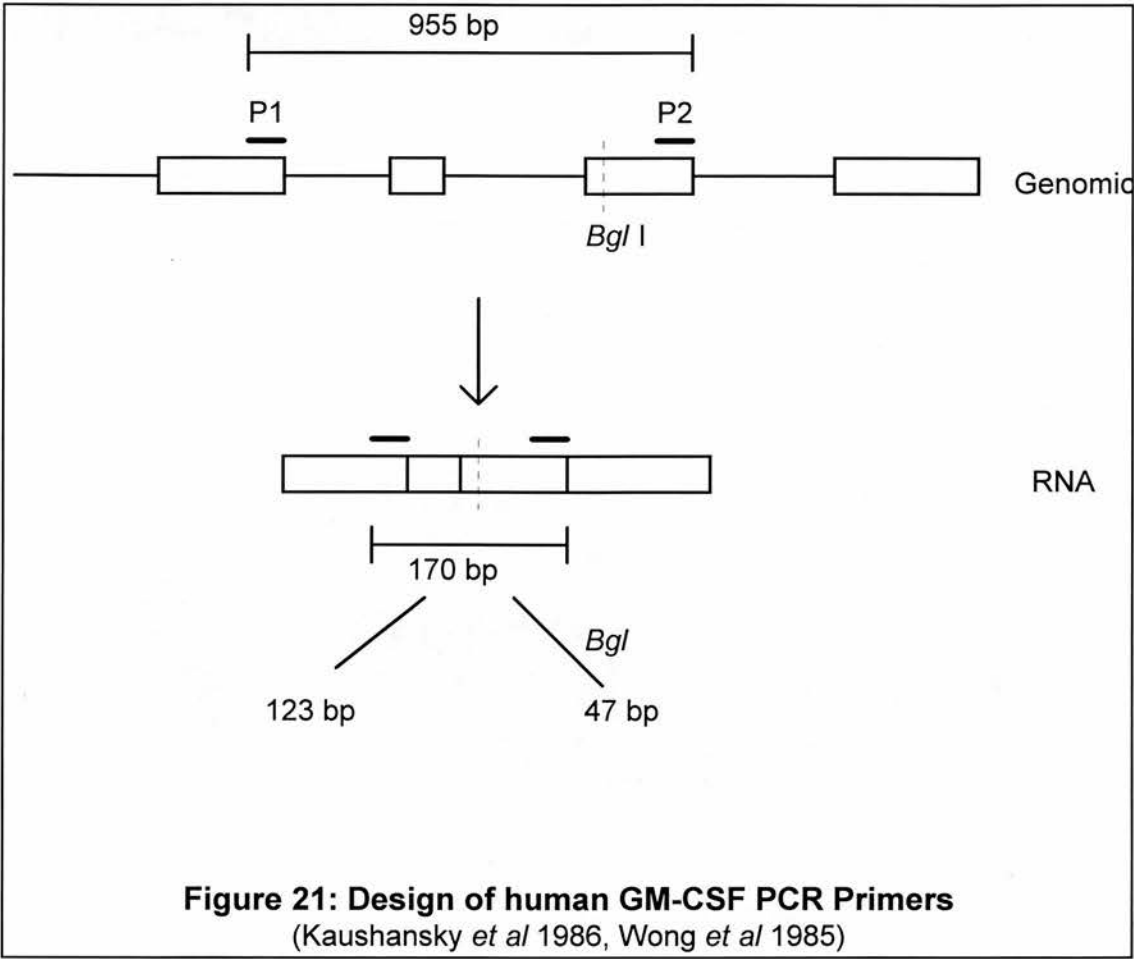
Total RNA was extracted from DU145, PC-3, LNCaP and human blood cells (used as a positive control). RNA was converted to cDNA by reverse transcription, and then specific primers were used to expressly amplify GM-CSF (illustrated in figure 21) and HPRT transcripts by PCR.

Plate 25 illustrates the PCR products from DU145 (A), PC-3 (B), human blood cells (C) and LNCaP cells (D). All cells showed the expression of the 280 base pair HPRT transcript product (lanes 1), which provided an internal, PCR control. DU145, PC-3 and blood cells all showed the presence of a 170 base pair product, the expected size of the GM-CSF transcript (lanes 2). These 170 base pair products could be specifically cleaved by *Bgl* 1 to produce two smaller fragments, 123 and 47 base pairs (lanes 3). This cleavage was calculated from the GM-CSF cDNA sequence (Wong *et al* 1985). The ability of *Bgl* 1 to produce these two fragments provides good evidence that GM-CSF transcripts had been amplified and were present in these cells. These results were identically produced on three separate RNA preparations.

The LNCaP cell line showed the expression of a 170 base pair product (figure d, lane 2). However, this could not be cleaved by *Bgl* 1 even if the enzyme concentration was increased six fold and the incubation time increased to 12 hours. This result was also reproducible.

Using the genomic sequence of human GM-CSF (Kaushansky *et al* 1986) the primers were designed to span two introns, therefore any genomic

contaminants (lanes 4) would produce 955 base pair products. Lanes 4 illustrate the presence of these contaminants in the PC-3, DU145 and blood cell RNA preparations, no such product was found in the LNCaP. This indicates that the 170 base pair products were due to the amplification of transcripts and not genomic DNA.



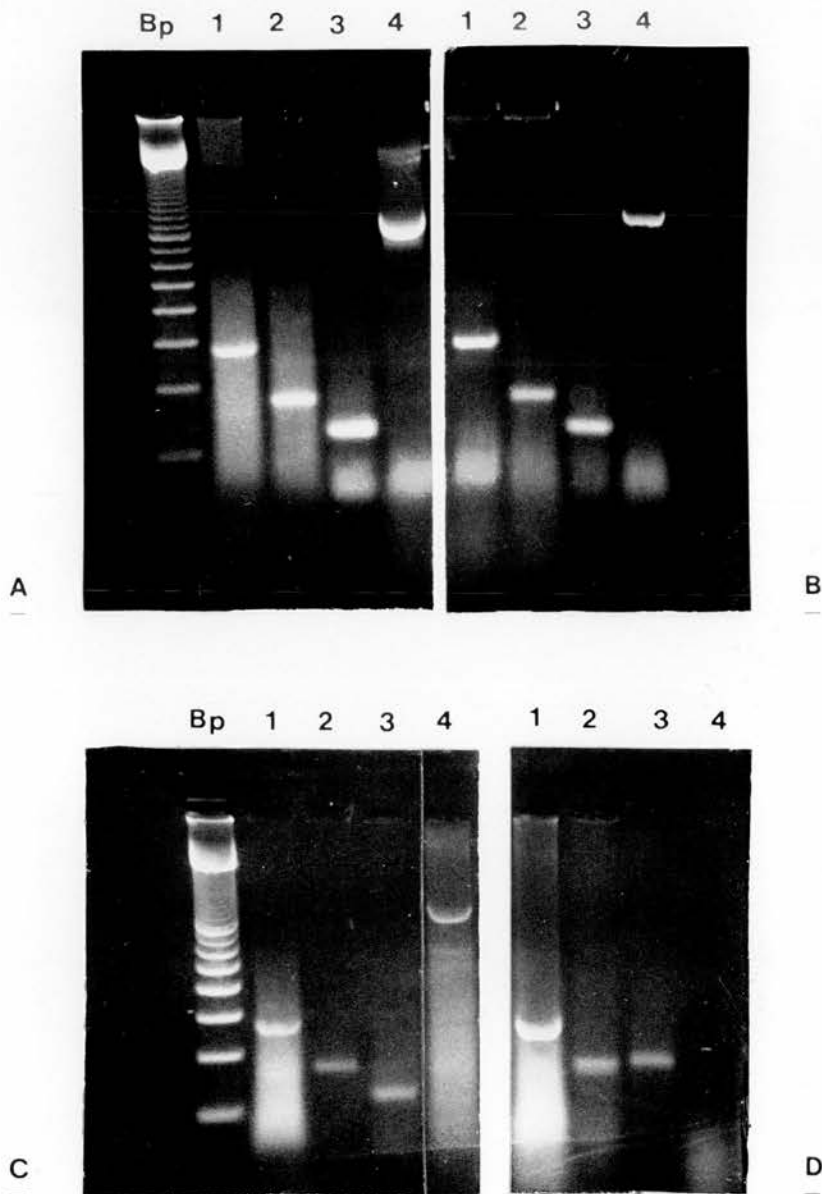


Plate 25: PCR amplification of GM-CSF and HPRT gene transcripts.

cDNA was prepared by reverse transcription of 1 μ g total cellular RNA from the cell lines DU145 (A), PC-3 (B), human blood cells (C) and LNCaP (D). This cDNA was amplified by PCR using primers for HPRT (lanes 1) and GM-CSF (lanes 2). GM-CSF products were specifically cleaved with *Bgl* I (lanes 3) and genomic contaminants are shown in lanes 4. The products were size fractionated on a 2% agarose gels together with 100 base pair markers (Bp).

CHAPTER 4

DISCUSSION

Malignant prostate cells grow slowly in the primary tumour, but, once they metastasise to the bone, the growth rate increases. These metastases are often destructive and therefore pose a greater health risk to the patient than the primary tumour. 84% of prostate cancer patients develop skeletal metastases (Berretoni & Carter 1986) which form osteoblastic lesions. Batson (1940) first suggested that the formation of skeletal metastases could be explained by the mechanical seeding of prostate cancer cells via the vertebral venous system. However, this theory on its own does not explain the increased growth rate of prostate cancer cells at secondary skeletal sites. One explanation for this increased growth, is the presence of prostate mitogens in the bone environment. In this way a preferential site would be provided for tumour development, in comparison to other locations in the body. Similarly, researchers have attributed the formation of osteoblastic lesions to growth factors secreted by the invading prostate tumour cells. Consequently, paracrine growth factors acting between osteoblasts and prostate cancer cells may play an important role in the formation of prostate skeletal metastases.

This study has concentrated on looking for soluble factors present in the bone environment that are mitogenic for human prostate cancer cells. This was investigated by assessing the effects of the following growth factors or media on the growth of both hormone sensitive (LNCaP) and insensitive (DU145 and PC-3) prostate carcinoma cell lines:

- a) media conditioned by osteoblast-like cells

- b) media conditioned by red bone marrow from the proximal femur
- c) media supplemented with recombinant haematopoietic growth factors

Finally, the ability of prostate cancer cells to produce GM-CSF was investigated, to ascertain if such a capability was important to the formation of a skeletal metastasis.

Media Conditioned by Human Osteoblast-like Cells Stimulates the Growth of Prostate Cancer Cell Lines.

The effect of soluble factors secreted by primary cultures of osteoblast-like cells on prostate carcinoma cell line growth was examined. Osteoblasts were cultured with a method, known from previous investigations, to produce cells with osteoblast-like characteristics (Beresford *et al* 1983, 1984, 1986). For the purpose of this thesis, therefore, osteoblast-like cells were characterised by their ability to increase alkaline phosphatase production in response to 1,25 (OH)₂ vitamin D. This was demonstrated by histochemical staining and by colourimetric assay.

The studies presented within this thesis indicated that media conditioned by primary cultures of human osteoblast-like cells could significantly ($p < 0.05$) stimulate the growth of the prostate cell lines DU145, PC-3 and LNCaP in a dose dependent manner. This is a novel result and found to be reproducible with a wide range of different osteoblast-like cell cultures. This stimulatory activity showed some specificity to the osteoblast since media conditioned by skin fibroblasts had no effect on the growth of DU145 or PC-3 cells. This also suggested further that the stimulatory effects of OBCM were due to osteoblast-like cells, since both the osteoblast and skin

fibroblast derive from connective tissue fibroblasts (Beresford *et al* 1983, 1984,1986).

The cell lines used demonstrated different responses to the mitogens in OBCM. PC-3 cell growth was consistently (and significantly) stimulated by OBCM to a greater extent (3.2-3.9 fold) than DU145 cells (1.9-2.4 fold), when compared to growth in serum free medium. The LNCaP cell line displayed only a 1.3-1.5 fold stimulation of growth due to OBCM. This decreased sensitivity maybe due to a number of factors:

a) The presence of FCS in the experimental medium may mean the growth of LNCaP cells was already stimulated before the addition of OBCM.

Therefore FCS may mask any potential stimulatory effects of OBCM at lower doses.

b) FCS may contain factors which are inhibitory to the prostate mitogens in OBCM.

c) LNCaP cells may secrete factors which are antagonistic to OBCM mitogens.

d) LNCaP cells may be less responsive to OBCM, because they possess fewer receptors to the osteoblast mitogens, or a post-receptor abnormality.

Some of these problems could be answered if PC-3 and DU145 cells were cultured under exactly the same conditions as the LNCaP cell line (i.e., with FCS). If the growth stimulation then became depressed in the presence of FCS, this would suggest that FCS does mask the stimulatory effects of OBCM. However, if the growth stimulation remained greater than that of LNCaP this would imply that LNCaP cells have decreased sensitivity to OBCM.

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A difference in sensitivity of the three cell lines may have arisen due to differences in the number and (or) type of mitogen(s) receptors, present on each cell line. Prostate cell line growth has already demonstrated varying sensitivity to TGF β for such reasons. Experiments have shown that TGF β inhibits the growth of DU145 and PC-3 cells but does not affect LNCaP growth (Wilding *et al* 1989b). Although this effect is inhibitory, the pattern is the same as for OBCM i.e., PC-3 and DU145 growth was affected whereas LNCaP growth was unaffected. These differential responses were attributed to the presence of TGF β receptors on PC-3 and DU145 cells but not on LNCaP. PC-3 cells possessed more binding sites than DU145 cells and were also inhibited to a greater extent than DU145 cells. This illustrates how the presence or absence of growth factor receptors can dictate the growth response of a cell.

Characterisation of OBCM

Pulsing the osteoblast-like cells with DHT or 1,25 (OH) $_2$ Vitamin D did not influence the production of prostate mitogens, nor had these two hormones any synergistic effects with the mitogens, on prostate growth. The prostate mitogenic activity induced by OBCM was however inhibited by heat and acid.

Since high performance chromatography techniques were unavailable, OBCM was crudely fractionated with 'Centricon filters'. This procedure suggested the presence of two or more active components, with molecular weights of >100Kd and 30-100Kd. To test the efficiency of filtering, the various molecular weight fractions were run on a polyacrylamide gel and analysed by silver staining. This indicated that filtration was not 100% efficient. The staining revealed that some proteins with molecular weights less than that of their filter cut-off point were retained on the filter. This meant that

Discussion

the decrease in growth stimulation observed when DU145 cells were incubated in the OBCM fraction '<100Kd' compared to '>100Kd' could have been due to:

- a) the absence of stimulatory factors >100Kd (efficient filtering) or
- b) the absence of stimulatory factors <100Kd, because they were retained on the filter (inefficient filtering).

If the effect of (b) is a simple dilution of the stimulatory factors '<100Kd' and not a loss of unknown paracrine interactions, then this should not affect any stimulatory activity of the '<100Kd fraction'. This is presumed because the dose response analysis of OBCM indicated that significant stimulation of DU145 cells was achieved with concentrations of OBCM between 6.25-50%. Since 50% OBCM was filtered, it would have to be greatly diluted (by retention) to influence its stimulatory activity. Therefore the decrease in DU145 stimulation in the fraction '<100Kd' is most likely due to loss of stimulatory factors greater than 100Kd and not due to loss (dilution) of factors <100Kd, i.e., the filters were accurate.

These results suggest that growth factors with molecular weights less than 30Kd are unlikely to be the prostate mitogens in OBCM. With additional time these studies could be confirmed by further fractionating the OBCM with high performance liquid chromatography.

Site Specificity

The importance of osteoblast-derived factors in the production of prostate skeletal metastases was investigated by examining the effects of factors from other potential prostate metastatic sites. This was assessed by the response of prostate cells to media conditioned by cell lines derived from kidney, lung and bladder. The results demonstrated no stimulation of the prostate cells by any of the three media (at 50% dilution). In support of these

results, Charkal-Roy *et al* (1989) could find no significant response of PC-3 cells to media conditioned by human kidney, muscle or skin tissue. Therefore prostate cell lines were more responsive to soluble factors produced by osteoblast-like cells than factors produced by kidney, lung or bladder cell lines. This characteristic may be a contributory factor to the metastasis of prostate cancer to bone.

In similar, but reversed experiments the effects of exposing 50% OBCM to cell lines derived from other cancers, metastasising to bone was analysed (bladder, liver and breast). The results indicated that OBCM had no effect on the growth of these cell lines, whilst still stimulating prostate cells. Cancers of the bladder, liver and breast usually produce osteolytic lesions. If this is a characteristic shared by their respective cell lines, then it may indicate that the metastasis of prostate cancer to bone depends not only on growth factors from the bone environment but also from the prostate cancer cell. Further research on this preliminary data would help to establish whether or not the selective metastasis of prostate to bone shows a dependency on bi-directional paracrine effects between the osteoblast and the prostate cancer cell. This study should include examination of media conditioned by a wider range of cell lines, tumours and primary cultures derived from both osteolytic and osteoblastic tumours.

Media Conditioned By Bone Marrow From Proximal Femora Has No Effect On Prostate Cell Line Growth.

In addition to the studies on osteoblast-like cells, the effects of bone marrow (derived from the same bone samples) on prostate cell line growth, were also investigated.

Studies by Charkal-Roy *et al* (1989) have already shown that media conditioned by bone marrow removed from infant sternum can stimulate the growth of prostate cell lines, DU145 and PC-3. Later experiments attributed some of this stimulatory activity to transferrin (Charkal-Rossi *et al* 1992). Other researchers (Klose *et al* 1987) have however demonstrated that rat BMCM inhibits the growth of rat prostate tumours.

To try and clarify the effect of BMCM on prostate cancers, this study examined the effect of media conditioned by red bone marrow (from the proximal femur) on the growth of prostate carcinoma cell lines. It was found that both DU145 and PC-3 showed no growth response to this red BMCM, if it was diluted 2 fold with SFM. This concentration of BMCM was used because Charkal-Roy *et al* (1989) showed this dose caused maximal stimulation. Additionally, our own studies with OBCM indicated that 50% conditioned medium gave maximal response. Since Charkal-Roy *et al* (1989) supplemented their cell growth experiments with 1% FCS (v/v), it was tested whether or not supplementing the BMCM of these studies with 1% and 5% FCS (v/v) could also produce stimulatory responses. However, the prostate cell lines still showed no response to the BMCM collected in this study when supplemented with FCS.

There are several factors which may account for the discrepancies between this work and that of others, regarding the effects of BMCM on prostate cancer cell growth. These are as follows:

- i) Species
- ii) Presence of transferrin
- iii) Age
- iv) Activity of bone marrow

Each of these will now be discussed in further detail.

Firstly, the inhibitory effects of rat BMCM could be an effect confined to the rat species (and previously discussed in the introduction). Secondly, the studies by Charkal-Rossi *et al* (1992) demonstrating that transferrin was one of the stimulatory components in their BMCM, may affect the results of this study. They showed that maximum stimulation was reached with 10µg/ml of transferrin. In view of the fact that the serum free culture technique employed in this thesis contained 10mg/ml transferrin it seems likely that this level would mask the stimulatory effects of this factor already present in the BMCM. However, transferrin only accounts for part of the stimulatory response induced by Charkal Roy's BMCM, therefore other factors must also play a part.

There is a large difference in the age of the bone marrow used in both studies. The red bone marrow in this thesis was predominantly obtained from the proximal femora of elderly patients having hip replacements, whereas Charkal-Roy's red marrow came from sternal fragments of children having corrective surgery. The disease state of bone marrow from hip replacements should not affect the results since hip replacements are usually required because of arthritis. Arthritis is a disease which does not directly involve bone cells or bone marrow but is caused by the loss of calcium and cartilage, leading to bone thinning. Potentially therefore, the age of the bone marrow may be a contributing factor. The site from which the bone marrow was removed may also be important. In the adult, the active sites of the bone marrow are the proximal ends of the femora and humeri, the sternum, clavicle, vertebrae, ribs and skull (Harrison 1981), which are also major sites for prostate skeletal metastases (Jacobs 1983). This implies that prostate cancer spreads to sites of active bone marrow. The bone marrow in Charkal-Roy's (1989) studies came from the sternum, whereas the bone marrow for

these studies came from the proximal femur. Generally the bone marrow of adult long bones is inactive therefore the advanced age of the specimens used in this study may mean the marrow was inactive. The idea that active bone marrow is important for stimulation is further reinforced by results from Charkal-Roy *et al* (1989) who found no stimulation of prostate growth by media conditioned with yellow bone marrow (haematopoietically inactive). Therefore, stimulation of prostate cancer in the bone environment may be due to the production of soluble mitogens from active bone marrow.

Alternatively, as prostate cancer only manifests in elderly males and therefore the use of bone marrow from a child is less valid than the use of marrow from an adult. Potentially therefore, the lack of stimulation seen by the BMCM in this thesis is a more accurate portrayal of events *in vivo*, in comparison to the results of Charkal-Roy *et al* (1989). This raises the possibility that the stimulation of prostate cancer in the bone environment is due to osteoblast derived mitogens and not bone marrow.

It is suggested that further work should investigate the following aspects of BMCM stimulatory activity:

- 1) Using the BMCM prepared, as described in this thesis, diluted in serum free media without transferrin, the effects of transferrin in BMCM on prostate cell line growth could be assessed.
- 2) Assess how prostate cell line growth is affected by the age or activity of bone marrow.

These experiments may help elucidate whether BMCM stimulates prostate cell lines due to transferrin alone or due to other factors produced by active bone marrow, or not at all.

The Response Of Prostate Carcinoma Cell Lines To Haematopoietic Growth Factors

Bone marrow and osteoblasts are rich in haematopoietic growth factors, and since earlier studies have demonstrated that some of these HGF can stimulate the growth of non-haematopoietic malignant cell lines (Dedhar *et al* 1988, Block *et al* 1992, Young *et al* 1992, Berdel *et al* 1992), it was decided to examine their effects on prostate carcinoma cell lines.

The studies presented show that medium supplemented with rGM-CSF (0.01-100 IU/ml) stimulated the growth of DU145 and PC-3 cells in a dose dependent manner. The growth stimulation could be inhibited with a monoclonal antibody raised specifically to human GM-CSF, suggesting that the prostate stimulatory activity was specific to GM-CSF. The growth of LNCaP cells was stimulated at doses of rGM-CSF >5IU/ml, which indicated decreased sensitivity in comparison to DU145 and PC-3. The reasons for this decrease in sensitivity may be similar to those for OBCM having less effect on LNCaP growth and could therefore be investigated in a similar manner. Other authors have indicated decreased sensitivity to GM-CSF, with increasing FCS concentration (Dedhar *et al* 1988, Berdel *et al* 1992). Serum therefore appears to be the most likely reason for the decreased response of LNCaP cells to rGM-CSF, though this remains to be made clear.

Medium supplemented with rEPO (0.1-100mIU/ml) stimulated the growth of DU145 and PC-3 cells. This conflicts with earlier studies by Berdel *et al* (1991) who found no response from a prostate carcinoma cell line 'B' to rEPO (0.01-100U/ml). The cell line was defined as being derived from a prostatic carcinoma in their own laboratory. Therefore the characteristics of 'B' may be different to those of PC-3 or DU145 and this could account for the

differences in growth response to rEPO. In the same study Berdelet *et al* (1991) found no effect on the clonal growth of a wide range of cell lines derived from human tumours in response to rEPO (e.g., lung, breast, stomach, renal, ovarian and colorectal). Inhibition of the biological activity of rEPO with a monoclonal antibody was not attempted because at the time these experiments were conducted there was no suitable antibody.

The stimulation of prostate cancer cell lines by rEPO and rGM-CSF lends support to the findings of other groups who have similarly shown that the growth of non-haematopoietic malignant cell lines (breast, bladder, lung, colorectal) can be stimulated by the haematopoietic growth factors, GM-CSF, IL-3 and G-CSF (Dedhar *et al* 1988, Nakata *et al* 1991, Block *et al* 1992, Young *et al* 1992, Berdel *et al* 1992). Much controversy exists in this field of investigation since other research groups have found no such stimulatory activity in response to the haematopoietic growth factors (Foulke *et al* 1990, EORTC 1991, Twentyman & Wright 1991, Guillaume *et al* 1993). These latter groups however often lacked (Guillaume *et al* 1993) or presented unconvincing (EORTC 1991) positive controls. The positive controls presented in the paper by the EORTC indicated significant stimulation of growth when exposed to rGM-CSF. However in comparison to some of their claimed negative results, it is obvious that a number of these were stimulated to similar magnitudes, as the positive controls, and also possessed smaller deviations of error. This suggests that some of the non-haematological malignancies were significantly stimulated by rGM-CSF.

Inconsistent results probably arose due to the use of different cell growth assays (e.g., clonal growth, thymidine incorporation, cell counts metabolic CO₂ production, MTT assay). The EORTC group made some attempts to compare clonogenic, MTT and thymidine assays. They stated that

GM-CSF had no effect on MCF-7 (breast carcinoma cell line) when assessed by any of the three assays. The validity of this statement is however uncertain due their unconvincing positive controls (discussed above). Results may also vary due to different dose levels and sources of recombinant haematopoietic growth factors (e.g., Sandoz, Amgen, Behringwerke, Glaxo). Deviation in the characteristics of the same cell line between laboratories may also have occurred. All of the above variations in experimentation need to be evaluated in order to comprehend the real effect of haematopoietic growth factors on non-haematopoietic malignancies. The problem of divergent cell lines may be overcome by investigating *in vivo* experiments with athymic mice or by examining the effects of haematopoeitic growth factors on primary cultures of prostate cancer.

The studies presented have indicated that prostate cancer can be stimulated by haematopoietic growth factors. The presence of haematopoietic growth factors in bone marrow would therefore provide an advantageous site for the secondary growth of a tumour. This idea is supported by other experimental results. The lowest dose of rGM-CSF capable of stimulating PC-3 and DU145 cell growth was found to be 0.1IU/ml (equivalent to 10pg/ml). Results from ELISA experiments indicated that the bone cells (MG63 and osteoblast-like cells) produce 21-170pg/ml of GM-CSF-like material, which would be sufficient (i.e., >10pg/ml) to cause growth stimulation of prostate cancer cells. A review by Nicola (1989) stated that natural sources produce 10ng/ml of GM-CSF. Therefore bone that is haematopoietically active and producing quantities of GM-CSF as above, would provide a selective site for the growth of prostate cancer cells, allowing the development of a metastatic tumour. The possibility that prostate cancer is only stimulated by active bone marrow has already been raised. Since haematopoiesis is a process largely

controlled by haematopoietic growth factors, these growth factors may be more specifically involved in the selective metastasis of prostate cancer to active bone marrow.

This idea is further supported by the results of Charkal-Roy *et al* (1989) who demonstrated that combinations of haematopoietic growth factors (IL-3, M-CSF, G-CSF) could stimulate the growth of prostate carcinoma cell lines up to 50% of that induced by BMCM. Also, recent experiments, investigating IL-1 have found that this growth factor is important for the formation of secondary melanoma tumours in the bone marrow. Arguello *et al* (1992) demonstrated that the administration of IL-1 into mice increased the number of bone or bone marrow metastases formed from melanoma cells. This effect was mediated through the release of prostaglandins and haematopoietic growth factors such as GM-CSF.

Therefore, experimental evidence is growing to suggest that prostate cancer spreads to sites of active bone marrow because of the release of soluble mitogens such as the haematopoietic growth factors.

Can GM-CSF account for the OBCM stimulation of prostate cell lines?

The results reported in this thesis suggest that GM-CSF is produced by osteoblasts, a characteristic of these cells previously reported (Horowitz *et al* 1989). Whether or not this osteoblast-derived GM-CSF is responsible for the prostate stimulatory activity of OBCM is debatable. Pre-incubation of OBCM with concentrations of anti-GM-CSF mAb known to inhibit the rGM-CSF induced stimulation of DU145 cells, had no effect on the DU145 mitogenic activity of OBCM. Other experiments could not find any additive effects of DU145 growth stimulation when media containing EPO, GM-CSF or OBCM were combined. This may mean that OBCM already contains GM-CSF

and EPO. Immunological investigations showed that OBCM did contain GM-CSF-like material, however, it is unlikely that OBCM contains EPO, since EPO is a hormone normally produced by the kidney. However, the lack of additive effects when media containing rEPO or rGM-CSF were combined suggested that DU145 growth was instead stimulated to a maximum by all three test media. Since these two solutions were originally devoid of each other, an additive effect would have been expected if growth was sub-maximal. It is unlikely therefore that GM-CSF on its own can account for the prostate mitogenic activity of OBCM.

Potentially, GM-CSF is one of many factors produced by osteoblasts which act in a synergistic fashion to stimulate prostate cancer growth. Fractionation of the OBCM was carried out to try and clarify this. The two stimulatory fractions of OBCM ('>100Kd' and '<100Kd') indicated no inhibition of growth stimulation when incubated with the anti-GM-CSF mAb. Since GM-CSF has a molecular weight of 22Kd (Wong *et al* 1985) and the stimulatory fractions of OBCM had, approximately, molecular weights greater than 30Kd the likelihood of GM-CSF being responsible for OBCM stimulatory activity is less likely. However, analysis of OBCM for the presence of GM-CSF by Western Blot indicated GM-CSF-like material (and recombinant GM-CSF) to have a molecular weight of 42Kd. If this was due to dimerisation or an interaction of GM-CSF with SFM then GM-CSF would appear in the stimulatory fraction 30-100Kd.

The results presented have not been able to demonstrate whether or not GM-CSF is in part responsible for the prostate stimulatory activity of OBCM. However since both immunohistochemistry and ELISA suggested the presence of GM-CSF-like material in OB cultures, it is likely to contribute to this effect. The GM-CSF-like material produced by these cultures was not

shown to have any biological activity therefore it cannot be ruled out that the GM-CSF-like material was inactive.

Research has shown that Lewis-lung carcinoma cell lines can produce GM-CSF (Young *et al* 1992). Therefore media conditioned by another lung carcinoma cell line, HF19, might be expected to stimulate the growth of the prostate carcinoma cell lines. Our immunohistochemical studies have indicated that the lung cell line, HF19, was immunoreactive with an anti-GM-CSF mAb. However media conditioned by this cell line showed no evidence of stimulating PC-3 and DU145 cells. Unlike the experiments by Young *et al* (1992), we did not demonstrate that HF19 conditioned medium possessed GM-CSF biological activity, therefore there may not be any active GM-CSF in the medium. This would explain the lack of prostate cell line growth stimulation. Alternatively, since immunostaining is a very sensitive procedure the lung cells may be producing GM-CSF but not at great enough levels to stimulate the prostate cell lines, or there may be other growth factors in the HF19 conditioned medium which are antagonistic to the GM-CSF.

Secretion Of GM-CSF by Prostate Cell Lines

Experiments carried out by Young *et al* (1992) have shown that lung carcinoma cell lines can not only respond to GM-CSF but also secrete this haematopoietic growth factor. Further studies were therefore conducted to investigate whether or not GM-CSF was produced by prostate cell lines.

Immunohistochemical analysis, showed immunoreactivity of anti-GM-CSF mAb with DU145 and PC-3 cell cultures. The cell staining was specific to the perinuclear region which is indicative of a secretory protein. This was assumed because exogenous GM-CSF bound to its receptor would produce

an even staining, not one confined to regions of the Golgi. The LNCaP cell line showed no staining. In support of these results, osteoblast-like cells and MG63 demonstrated positive staining. These two cell types were used as positive controls because both have osteoblast characteristics, and osteoblasts are known to secrete GM-CSF (Horowitz *et al* 1989). To demonstrate the specificity of the anti-GM-CSF mAb, the positive stain of DU145 cells was inhibited by pre-incubation of the antibody with excess rGM-CSF. Pre-incubation of the antibody with either rIL-3 (which shares the same β receptor subunit as GM-CSF [Kitamura *et al* 1991]) or rEPO, did not affect the positive stain, indicating further the specificity of the antibody.

Prostate cancer primary cultures of both epithelial and fibroblastic cells were immunostained with the anti-GM-CSF mAb. Epithelial cells showed positive staining only. Although this result was reproduced on three different prostate cancer tissues, the study would be more conclusive with greater a number of tissue samples. A selection of malignant cell lines other than prostate cancer (available in our laboratory) were similarly immunostained. Positive staining was seen in melanoma and lung cell lines, these results confirm those already reported by Young *et al* 1992. Negative results were found for bladder, liver, osteosarcoma and larynx.

Media conditioned by the prostate cell lines was analysed by both ELISA and Western blotting. These techniques indicated that GM-CSF-like material was produced by both PC-3 and DU145 cells but not LNCaP. The Western blot revealed a protein band which migrated with recombinant GM-CSF. The protein band ran greater, unexpectedly. The correct molecular weight is 18-22Kd (Wong *et al* 1985). This is unlikely to represent a partially cleaved precursor protein, or state of glycosylation since the protein bands migrated at the same molecular weight as the recombinant material. A dimer

is unlikely since the blot was carried out under reducing conditions. Nevertheless because the molecular weight is that of a dimer it cannot be ruled out. It is possible that an adduct had formed between GM-CSF and BSA or some other component of the medium.

To complement the immunological studies, GM-CSF gene expression in the three prostate cell lines was investigated by RT PCR of total cellular RNA. GM-CSF gene transcripts were identified in PC-3, DU145 and blood cells. This was known from the presence of the expected 170bp product in all three cell types, which could be specifically cleaved by the *Bgl* 1 enzyme. The products (and primers for PCR) were all calculated from the GM-CSF cDNA sequence (Wong *et al* 1985, Kaushansky *et al* 1986). The LNCaP cell line showed expression of the 170bp product but since this could not be cut with a *Bgl* 1 enzyme, whether or not it was a GM-CSF gene product remains uncertain. Since the primer sequences were long and therefore very specific it is unlikely that a non-specific band occurred at 170bp. It may be that the T_4 DNA polymerase introduced a mutant nucleotide into the *Bgl* 1 recognition site, though this is unlikely since the result was repeatable. It is more probable that the LNCaP cell line, used in our laboratory, already contains a mutation which makes it resistant to digestion. The presence of a mutation may also explain the absence of any GM-CSF protein. Further experiments could answer this problem by sequencing the LNCaP 170bp product or Southern blotting.

These results together provide strong evidence for the production of GM-CSF by the prostate cancer cell lines PC-3 and DU145. LNCaP cells showed no protein expression of GM-CSF and no conclusive gene expression.

Recent research by Guillaume *et al* 1993 demonstrated no GM-CSF gene transcription by DU145 cells which disagrees with the results presented here. They did however demonstrate the larynx cell line used in this thesis showed no GM-CSF gene transcription which is in agreement with the findings of this thesis. It seems likely that cytokine gene transcription is very variable within cell lines and may be a function of passage number and clonal changes. Pekarek *et al* 1993 have indicated that tumours of the same type and lineage can have distinct cytokine mRNA expression patterns. Therefore to correctly analyse cytokine expression in non-haematological malignancies one should investigate the malignant tissues themselves.

Immunoreactivity of Prostate Tissue Sections With a GM-CSF Monoclonal antibody.

To investigate the latter statement a number of prostate tissues of varying disease states were immunostained with the anti-GM-CSF mAb. Immunoreactivity of the antibody was found with poorly differentiated prostate cancers but not well differentiated ones. Staining was also absent in benign tissues. These results imply a connection between prostate cancer progression and GM-CSF production. Staining of the poorly differentiated cancer indicated positive staining only in areas of malignant epithelia and not in stromal sites. This supports the results found from the primary cultures, that GM-CSF-like material was produced from epithelial cells and not from fibroblasts.

It was interesting to note that normal prostate peripheral zone tissue indicated positive staining confined to basement membranes around the secretory duct epithelia. The explanation of this is unknown, but it may be attributed to the sequestration of GM-CSF-like material by glycosaminoglycans in the extracellular matrix, in a manner similar to that

seen within the bone marrow (Gordon *et al* 1987, Roberts *et al* 1988). The sequestering of haematopoietic growth factors in this way is thought to present them in a biologically active form for haematopoietic cells.

Again a greater number of prostate cancer tissue sections would help to confirm whether GM-CSF production is linked to cancer progression. The staining of actual metastatic tissue sections would help evaluate whether or not GM-CSF-like material is secreted at secondary metastatic sites.

Positive staining of the malignant prostate tissues did not necessarily mean these tissues produced GM-CSF. However the fact that DU145 and PC-3 cells showed the production of GM-CSF, by a number of experiments, strongly supports the idea that cancer tissues also produced GM-CSF.

Bi-directional Growth

In the prostate gland, malignancy arises from aberrant growth control. As discussed in the introduction, normal growth is under endocrine and paracrine growth factor regulation. Under androgenic control, growth factors from the stroma and epithelia interact so that the stimulatory effects of FGF and EGF are balanced by the inhibitory effects of TGF β (Schuurmans *et al* 1991, Steiner 1993). During malignant growth the prostate cells become androgen insensitive. Cells also become less sensitive to EGF and it is thought that they switch to an autocrine stimulation of growth by TGF α (Wilding *et al* 1989a, MacDonald *et al* 1990). Alteration of normal growth factor action may affect local paracrine growth controls which are highly dependent on one another. For example, the overexpression of TGF β 1 in prostate cancer (Steiner *et al* 1992) could affect the extracellular matrix production from stroma, leading to tissue breakdown or decreased cell

Discussion

adhesion (Lyons & Moses 1990). These effects may be responsible for the increasing propensity of a prostate cell to metastasise with increasing TGF β 1 production (Steiner *et al* 1992). In a similar manner, the production of GM-CSF-like material from malignant epithelia may have important growth or structural effects on the surrounding stroma. Alternatively, the interaction of GM-CSF with other growth factors may result in a more malignant phenotype.

Once the prostate cancer cell is able to metastasise to the bone, prostate growth factors may have important effects on the normal growth of the bone. The stimulation of prostate cancer cells in the bone environment and the production of osteoblastic lesions, suggests a bi-directional paracrine control of growth. The introduction of foreign mitogens from metastatic prostate cells in the bone environment would appear to interfere with the normal growth of the osteoblast causing a net increase in numbers. Conversely bone derived mitogens cause uncontrolled proliferation of prostate tumour growth. Increasing numbers of both cell types would accelerate the effect.

The high levels of GM-CSF produced by the PC-3 cell line (2.5pg/ μ g protein) may be implicated in the formation of osteoblastic lesions, since GM-CSF is a known stimulator of osteoblast growth (Evans *et al* 1989).

The release of latent TGF β from Dunning rat tumours has also been suggested as a potential factor causing osteoblastic lesions, since active TGF β can also stimulate osteoblast-like cells and inhibit osteoclasts (Matuo *et al* 1990, Chenu *et al* 1986). Recent results (Killian *et al* 1993) have indicated that latent TGF β is activated by prostate specific antigen (a serine protease specific to the prostate). This provides a unique mechanism for prostate cells to increase osteoblast growth.

There are several other growth factors which are released by prostate cancer cells and are capable of stimulating osteoblast growth. These include PDGF, IGF-I, IGF-II and FGF (Canalis 1981, Sitaras *et al* 1988, Canalis 1980, Matuo *et al* 1988, Rodan *et al* 1987, Story *et al* 1989). As yet, no-one has investigated these factors in relation to prostate-derived osteoblast mitogens.

Unknown prostate-derived osteoblastic mitogens have been identified by several researchers. Koutsilieris *et al* (1987) identified a 10Kd peptide produced from malignant tissue - obviously not GM-CSF nor TGF β , whose molecular weights are 22Kd and 25Kd respectively. Simpson *et al* (1985) identified a 20Kd protein encoded by an mRNA fraction of 1800 bases. This could be GM-CSF (encoded by 1200 nucleotide mRNA [Gough *et al* 1984]) but not TGF β (encoded by a 2500 nucleotide mRNA [Derynck *et al* 1988]). Either TGF β or GM-CSF could be responsible for the 26-30Kd PC-3 derived osteoblast mitogen, identified by Perkel *et al* (1990). However whether or not GM-CSF or TGF β were responsible for these unknown mitogens was not assessed.

The studies presented in this thesis, have indicated the potential for a bi-directional control of growth between osteoblasts and prostate carcinoma cells. The production of GM-CSF by prostate carcinoma cell lines can stimulate osteoblasts and conversely osteoblast-like cell derived mitogens can stimulate prostate cancer cells.

Gleave *et al* (1991) have already demonstrated such a bi-directional effect using nude mice injected with LNCaP cells. LNCaP tumours were induced most consistently when coinoculated with media conditioned by a bone fibroblast cell line. In vitro, LNCaP conditioned medium could stimulate the growth of the same bone fibroblast cell line. The results could be further investigated using primary cultures of osteoblasts. As briefly discussed,

analysis of bi-directional effects between cancers producing osteolytic or osteoblastic tumours and bone cells may help establish if prostate skeletal metastases are in part due to uncontrolled growth caused by paracrine growth factors of prostate cancer and osteoblasts.

Is GM-CSF Involved In Cancer Progression?

GM-CSF has been implicated in the aetiology of several cancers (Takeda *et al* 1991, Young *et al* 1992, Kohn *et al* 1993). For example, in lung carcinoma cell lines it may act as an autocrine growth factor (Young *et al* 1992). Prostate cell lines and prostate tissue sections all exhibited differing degrees of GM-CSF immunostaining. The more invasive cell lines produced greater amounts of GM-CSF and only the more malignant tissues indicated GM-CSF immunostaining. There were also variations in the sensitivity of the prostate cell lines to exogenous GM-CSF.

It has to be stressed that these results are preliminary, since both the number of tissue samples and cell lines do not allow the findings to be statistically significant. Although the invasive potential of our prostate cell lines was not investigated, previous research has indicated that the PC-3 cell line is more invasive than DU145 cells (Albini *et al* 1987, Kozlowski *et al* 1984). PC-3 cells produced more GM-CSF than DU145 cells (2.5pg GM-CSF/ μ g protein versus 1.7pg/ μ g) and they were also stimulated by rGM-CSF to a greater extent (i.e., 3 fold versus 2.3 fold). This would suggest that increased production of GM-CSF together with enhanced sensitivity (perhaps through increased numbers of GM-CSF receptors) leads to a greater capacity for autocrine growth and increased invasive potential. These results are supported by the fact that LNCaP cells which are the least invasive and

hormone sensitive, showed no production of GM-CSF-like material. Further evidence from the immunostaining of prostate tissue sections indicated that poorly differentiated prostate cancers are immunoreactive with an anti-GM-CSF antibody. The same was not true for well differentiated cancer or benign tissues. This too would imply that more malignant prostate cancers have a greater ability to produce GM-CSF. These facts indicate that the production of and the response to GM-CSF by prostate cancer may be connected to its progression.

It has been suggested that the progression of a cancer to a hormone insensitive state is assisted by the over expression of autocrine growth factors (King 1990, Eaton & Davies 1991). In light of this, the production of GM-CSF by prostate hormone insensitive cells (PC-3, DU145) but not by androgen responsive cells (LNCaP), may indicate that GM-CSF production aids the progression of prostate cancer to an hormone insensitive state. We could begin to understand whether the production of GM-CSF is linked to the loss of androgen sensitivity by a study of prostate cancer tissues which have undergone androgen ablation.

GM-CSF has been linked to cancer progression in other tumours. Takeda *et al* (1991) indicated that highly metastatic mouse tumours exhibit both GM-CSF gene expression and the ability to secrete GM-CSF activity into their culture medium. Non-metastatic mouse tumours had neither characteristic. Young *et al* (1992) also linked the production of GM-CSF to cancer progression in lung carcinoma cell lines. Using PCR analysis they showed metastatic variants could produce GM-CSF whereas non-metastatic variants could not.

M-CSF may also have a role in cancer progression. Results by Filderman *et al* (1992) demonstrated that the invasive potential of breast and

lung cell lines, expressing the *c-fms* oncogene product (mutant M-CSF receptor), could be increased by exposure to M-CSF.

How could GM-CSF Increase Invasiveness?

Since GM-CSF has been implicated as a growth factor involved in the progression of lung and now, prostate cancer, there must be a mechanism for this to occur. Several possibilities exist:

- a) Nerve growth factor-like proteins have shown a capacity to increase the mobility of prostate cell lines (Djakiew *et al* 1989). GM-CSF has also increased the mobility of melanoma cells (Kohn *et al* 1993) and Lewis lung carcinoma cells (Young *et al* 1992). Therefore secretion of GM-CSF by malignant prostate cells may increase their invasive potential by acting as a motility factor.
- b) It has been reported that the release of GM-CSF and IL-3 from Lewis lung carcinoma cells can induce myelopoiesis and the appearance of bone marrow derived immune-suppressor cells (Young *et al* 1991). Although no similar research has been carried out on prostate cancer, it can be seen that the secretion of GM-CSF by prostate cancer may have matching effects and allow the tumour cell to avoid immune attack.
- c) The secretion of various degradative enzymes and adhesive factors by prostate cancer cells has been linked to their invasive potential (Keer *et al* 1991, Pajouh *et al* 1991, Haq *et al* 1992). Since Lewis-lung carcinoma cells have shown an increased capacity to attach to and invade artificial basement membrane if they produce GM-CSF (Young *et al* 1992), a similar effect for the production of GM-CSF by prostate cancer should not be ruled out.
- d) TGF α is suspected to be an autocrine growth factor in prostate cell lines, since it is known to be both produced from and stimulate prostate cancer

cell line growth (Wilding *et al* 1989a). GM-CSF has the same prostate growth characteristics and therefore may also act as an autocrine growth factor. This idea will be discussed further in the next section.

Is GM-CSF An Autocrine Growth Factor?

GM-CSF has already been implicated as an autocrine growth factor in Lewis-lung carcinoma (LLC) cells (Young *et al* 1992). These studies showed that the invasive potential of LLC cells could be inhibited with an anti-GM-CSF antibody and increased with exogenous GM-CSF. Using PCR analysis, they found GM-CSF gene transcripts in the metastatic LLC cells.

The experiments of this thesis have shown that prostate cancer cell lines are stimulated by exogenous GM-CSF and are also capable of producing GM-CSF. High levels of anti-GM-CSF antibody (20µg/ml) suggested some inhibition of the basal growth of DU145 cells. Therefore GM-CSF could be acting as an autocrine growth factor in prostate hormone insensitive cell lines. It would be important for further studies to demonstrate that the GM-CSF-like material produced by these prostate cells possesses GM-CSF biological activity. The lack of basal growth inhibition by the anti-GM-CSF mAb may be due to the production of biologically inactive GM-CSF.

Other investigations with various prostate cancer models have indicated that both TGFβ1 (Steiner *et al* 1992) and TGFα (Wilding *et al* 1989a) act as potential autocrine growth factors. Both these are likely to be in the media conditioned by the prostate cell lines, but could not be the GM-CSF-like material, because the ELISA antibody showed no cross-reactivity with either.

GM-CSF Receptors

If GM-CSF is acting as an autocrine growth factor (or paracrine) the logical progression to the experiments carried out so far would be to show the

presence of receptors on the prostate carcinoma cell lines. The GM-CSF receptor has two receptor subunits α and β . The α subunit has low affinity and is specific to GM-CSF (Gearing *et al* 1989) whereas the β subunit is shared with IL-3 (Kitamura *et al* 1991). When both subunits are bound to GM-CSF it results in the formation of a 45Kd high affinity receptor.

Using ligand exchange analysis, the presence of GM-CSF receptors was demonstrated in melanoma cells (Baldwin *et al* 1991, Kohn *et al* 1993). The presence of both receptor units was also determined by RT PCR in cell lines derived from cancers of the lung, head, neck, ovary, stomach and kidney (Guillaume *et al* 1993). Both Guillaume *et al* (1993) and Baldwin *et al* (1991) stated that the presence of GM-CSF receptor on the cell lines did not confer the ability to proliferate in the presence of GM-CSF. However the results by Kohn *et al* (1993) indicated that the presence of GM-CSF receptors on melanoma cells increased their capacity to migrate in response to GM-CSF, an important characteristic for a metastatic cell. Guillaume (*et al* 1993) indicated that DU145 cells possess only the α subunit and not the β . Since they did not test whether the cells were responsive to GM-CSF it is uncertain as to whether the α subunit on its own can produce growth stimulatory effects. It is also possible that the DU145 cell line used in these experiments had different characteristics to the one used in this thesis, due to clonal divergence etc. The results of Guillaume *et al* (1993) indicated the presence of GM-CSF receptors on non-haematopoietic cell lines and therefore the potential for GM-CSF to affect a variety of other cancers.

Clearly, there is a need to examine the presence of GM-CSF receptors in prostatic cells and relate their presence or absence to the cells capacity to respond to exogenous and endogenous GM-CSF.

CHAPTER 5

CONCLUSION

A number of events are thought to be important in the formation of a site specific metastasis including: a) selective adhesion at the secondary site due to specific cell adhesion molecules (Blood & Zetter 1990, Johnson *et al* 1991, Haq *et al* 1992); b) tumour cell production of hydrolytic enzymes capable of degrading the secondary organ's extracellular matrix (Zetter 1990); c) the ability of a secondary organ to sustain and increase the growth of a tumour cell depending on the growth factors it secretes (Nicolson *et al* 1986, Charkal-Roy *et al* 1989).

The initial aim of this thesis was to investigate whether or not growth factors present in the bone environment can stimulate the growth of prostate cancer, and therefore promote the selective metastatic spread of prostate cancer to skeletal sites.

Media conditioned by osteoblast-like cells or supplemented with rGM-CSF or rEPO were all capable of stimulating the growth of prostate cancer cell lines compared to control levels. Whereas media supplemented with rIL-3 or rG-CSF had no effect on growth. Similarly media conditioned by bone marrow from proximal femora also had no stimulatory effect on prostate cell line growth. These results and those of others suggest that the growth of prostate cancer is stimulated by either

- i) osteoblast-derived mitogens and not bone marrow, or
- ii) osteoblast-derived mitogens and soluble mitogens from active bone marrow (Klose *et al* 1987, Charkal-Roy *et al* 1989, Arguello *et al* 1992).

Conclusion

The latter would help explain why prostate skeletal metastases are frequently observed at sites with active marrow, and further implicates haematopoietic growth factors in their formation.

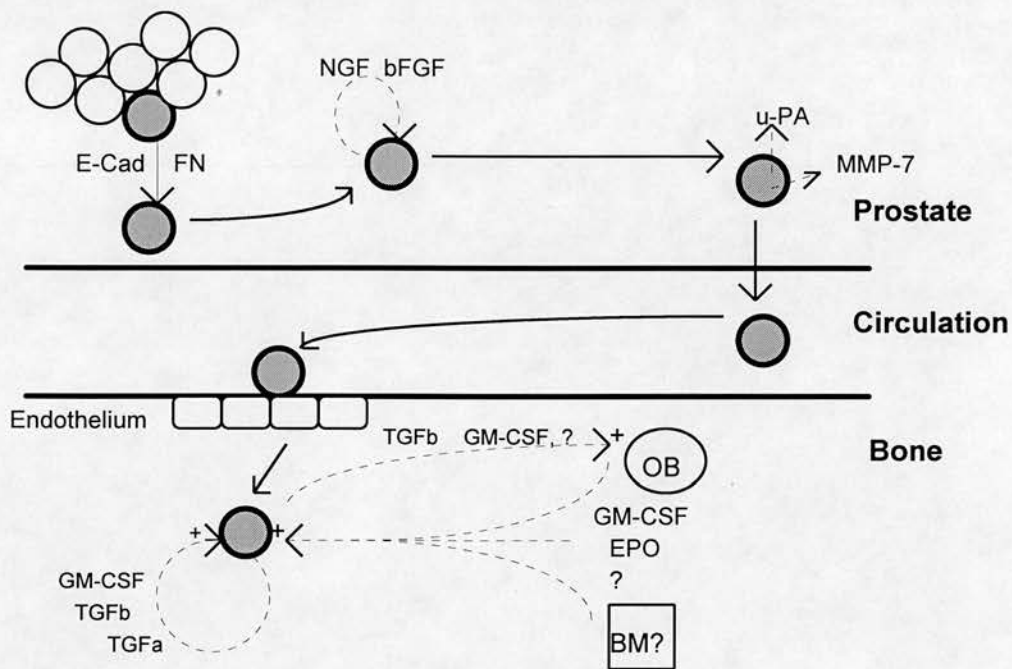
Studies were extended to investigate whether or not the prostate cell lines could secrete GM-CSF. DU145 and PC-3 (hormone insensitive) were both found to produce GM-CSF at both protein and RNA levels. LNCaP (hormone sensitive) cells did not show any GM-CSF protein production. Immunostaining of prostate tissue sections and primary cultures demonstrated that the production of GM-CSF-like material was restricted to epithelial cells and not stroma. Investigation of malignant prostate tumour sections showed the presence of GM-CSF-like material only in poorly differentiated cancers and not in well differentiated. These studies imply that GM-CSF production may be linked to the progression of prostate cancer, possibly as an autocrine growth factor. Previous research has shown that GM-CSF can stimulate osteoblastic growth (Evans *et al* 1989). Therefore the production of GM-CSF by prostate cancer indicates a potential mechanism for the formation of osteoblastic lesions.

Overall, the results demonstrated the growth stimulation of human prostate cancer cell lines by soluble osteoblast-derived mitogens, rEPO and rGM-CSF. These may be important factors required to establish prostate metastases at skeletal sites. The production of GM-CSF by prostate androgen insensitive, carcinoma cells could play a role in the formation of osteoblastic lesions and may represent an autocrine mechanism of growth.

Overview

Using the findings of this thesis and those of others (discussed in the introduction) a metastatic cascade was drawn (figure 1) to illustrate how prostate cancer may hypothetically spread to bone.

Figure 1: The Metastasis of Prostate Cancer To Bone



KEY:

- Normal Prostate Cell
- Malignant Prostate Cell

- | | | | |
|-----------|---|----|---------------|
| FN | - Fibronectin | OB | - Osteoblast |
| E-Cad | - E-Cadherin | BM | - Bone marrow |
| NGF | - Nerve growth factor-like protein | | |
| u-PA | - urokinase type plasminogen activator | | |
| MMP-7 | - matrix metalloproteinase-7 | | |
| TGF a & b | - Transforming growth factor alpha & beta | | |
| bFGF | - basic fibroblast growth factor | | |

Conclusion

Prostate cancer cells detach from the primary tumour due to decreased levels of the cell adhesion molecules, fibronectin and E-cadherin (Schalken *et al* 1988, Bussemakers *et al* 1992). They become mobile due to the secretion of motility factors such as nerve growth factor-like peptides or bFGF and invade the circulation through the release of urokinase type plasminogen activator and matrix metalloproteinase-7 (Djakiew *et al* 1993, Pienta *et al* 1991, Hoosein *et al* 1991, Pajouh *et al* 1991). The malignant cells evade the immune system by methods unknown and eventually adhere to bone endothelial cells (Haq *et al* 1992). Within the bone environment the tumour cells are stimulated by soluble osteoblast-derived mitogens, EPO and GM-CSF. Potentially they are also stimulated by soluble bone marrow-derived mitogens, including transferrin and other factors unknown (Charkal-Roy *et al* 1989, Charkal-Rossi *et al* 1992). The increased growth of prostate tumour cells is enhanced by the autocrine stimulation of GM-CSF, TGF α and TGF β (Wilding *et al* 1989a, Steiner *et al* 1992). Additionally, the production of GM-CSF, TGF β and unknown mitogens will also act in a paracrine fashion to stimulate osteoblast growth (Matuo *et al* 1990). Although some of the steps detailed are now well established others need to be confirmed.

Implications For Therapy

The concentrations of haematopoietic growth factors used in the experiments of this thesis were based upon those used in clinical treatments. Haematopoietic growth factors are used to treat refractory anaemia and for leukocyte recovery after cytotoxic chemotherapy (Oster *et al* 1990, Moore 1990). The results presented have shown that certain haematopoietic growth factors can stimulate prostate cancer cell lines. This raises concern for the

Conclusion

use of rGM-CSF following chemotherapy and the use of rEPO in managing refractory anaemia in prostate cancer patients. Further research into their use in cancer therapy is essential. There is a need to evaluate the effects of using GM-CSF to revive bone marrow after chemotherapy of prostate cancer patients in order to assess whether it is also encouraging the re-emergence of skeletal metastases. This concern applies to all cancers treated with haematopoietic growth factors, since mounting evidence indicates that these growth factors do have roles to play in non-haematopoietic malignancies. Further investigations are required to substantiate the preliminary evidence that EPO can stimulate prostate cancer cell lines.

The results presented herein indicate that manipulation of GM-CSF stimulatory activity within the bone environment might provide a therapy for reducing prostate skeletal metastases.

Future Work

The results raise three major questions which require further research in order to answer and develop these ideas.

1) Does prostate cancer metastasise to bone because of osteoblast factors alone or also because of factors derived from active bone marrow?

Primarily this question could be investigated by pathological observation of the exact sites that prostate skeletal metastases arise. Media conditioned by bone marrow from a variety of skeletal sites and ages would also require analysis. If prostate cell lines were stimulated only by active bone marrow, the mitogenic activity should be fractionated to evaluate whether it arises from transferrin alone or also from haematopoietic growth factors.

Conclusion

Finally an investigation of whether the selective metastasis is aided by a bi-directional enhancement of growth between the osteoblast and the prostate cancer cells should be undertaken. This could be investigated by the analysis of growth effects between osteoblasts and cells derived from cancers producing osteolytic lesions compared to those producing osteoblastic lesions.

2) Secondly, further investigation into whether the production of GM-CSF by prostate cancer is related to malignant progression should be confirmed by immunostaining a wider range of malignant and benign prostate tissues. If an association was found between prostate cancer progression and GM-CSF production this could be verified by RT PCR of fresh tumours. The use of in situ hybridisation could identify GM-CSF gene transcripts in the same tissue slides as those used previously for immunostaining. GM-CSF production in fresh tumours or media conditioned by primary cultures could be quantified by ELISA.

3) Finally, the presence of GM-CSF receptors on either prostate cancer cell lines or malignant tissue should be investigated. RT PCR would provide a means of initially identifying the subunits, and binding studies could be accomplished by ligand exchange assay. Both α and β subunits should be examined and the presence of one or both related with the sensitivity of the cell to exogenous GM-CSF.

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